

KIN DISCRIMINATION IN JUVENILE ATLANTIC
SALMON (*SALMO SALAR*) AND BROOK TROUT
(*SALVELINUS FONTINALIS*):
RECOGNITION CUES AND FUNCTION

CENTRE FOR NEWFOUNDLAND STUDIES

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KIN DISCRIMINATION IN JUVENILE ATLANTIC SALMON
(*SALMO SALAR*) AND BROOK TROUT (*SALVELINUS FONTINALIS*):
RECOGNITION CUES AND FUNCTION

By

© RUPIKA SUBASHINI RAJAKARUNA

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ABSTRACT

The ability to recognize relatives permits individuals to discriminate their kin, thereby enhancing their inclusive fitness. Many animal species, including salmonids, have the ability to recognize and discriminate kin from unrelated conspecifics. I conducted a series of experiments to examine the effect of recognition cues of genetic and environmental origin in juvenile Atlantic salmon (*Salmo salar*) and brook trout (*Salvelinus fontinalis*) and how kin association influences the growth of these species. In the first study (Chapter 2) I investigated the effect of diet as an environmental cue on the kin discrimination ability and found that kin discrimination in both Atlantic salmon and brook trout is influenced by dietary cues. Test fish could not discriminate kin and non-kin when the kin group was fed with a different diet and the non-kin group was fed with a similar diet. As second study, Chapter 3 dealt with a technique of isolation and characterization of MHC class II B1 locus and a brief survey of polymorphism of this locus in Atlantic salmon and brook trout collected from four different areas in Newfoundland. A high level of polymorphism both at the allelic level and in the amino acids is maintained at the MHC class II B1 locus in the two species. Using this technique I determined the genotype of kin and non-kin groups of both species and studied the influence of MHC class II B1 locus on their kin discrimination (Chapter 4). I found that MHC class II B1 locus significantly influence kin discrimination in juvenile Atlantic salmon and brook trout. The preference for individuals sharing alleles demonstrated that discrimination is taking place matching at the MHC locus. Data from the same study provided evidence for matching of the overall phenotypic similarity during

discrimination. Moreover, test fish could not discriminate kin and non-kin when the kin group did not share any alleles and the non-kin group shared both alleles at the MHC locus. In the fourth study (Chapter 5) I examined the interaction of the genetic and environmental cues used in kin discrimination in juvenile Atlantic salmon. Both environmental and genetic cues were found to be equally important and the relevance of each cue is context dependent. The last experimental chapter (Chapter 6) examined the effect of kinship on growth and demonstrated that higher and less variable growth occurred in individuals reared with kin compared to individuals reared with non-kin.

Taken together these data suggest that both genetic and environmental cues are important in kin discrimination the interaction of these cues is crucial for many cases of kin discrimination. Moreover, being cooperative and less aggressive towards kin result in direct and indirect fitness benefits to the individual.

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CHAPTER 1

GENERAL INTRODUCTION

Social behaviour involves the behavioural interactions among members of the same species or/and different species. All social behaviour involves communication, which is the passing of any information from one animal to another by means of evolved signals. The ability to recognize and discriminate among individuals is a prerequisite of most kinds of behaviours (Wilson 2000). The type of social behaviour examined in this thesis is the discrimination among conspecific individuals based upon their genetic relatedness.

Hamilton's (1964) kin selection model provides a general explanation for the evolution of social behaviour. He used the Wright's coefficient of relatedness (r) as the measure of the proportion of replica genes in a relative, and generalized the circumstances in which relative-helping of various sorts would evolve into the equation: $r b - c > 0$. According to this equation it was predicted that animals will favour closer relatives over more distant ones for any given act of helping and also that help to more distant individuals would only occur where the benefit (b) gained by the recipient outweighs the cost (c) to the altruist. He conceptualized a quantity 'inclusive fitness' which incorporates the maximizing property of Darwinian fitness. Inclusive fitness is the sum of an individual's own genetic fitness (direct fitness) plus all of its influence on the genetic fitness of its relatives (indirect fitness; Grafen 1982; Wilson 1987). Kin recognition is considered an important prerequisite of maximizing the potential for inclusive fitness benefits (Hamilton 1964; Wrangham 1982; Wilson 1987).

Kin recognition has been defined as 'the process by which individuals assess the genetic relatedness of conspecifics to themselves or others based on their perception of traits expressed by or associated with these individuals (Waldman *et al.* 1988). Kin recognition is an unobservable internal process and the exhibition of differential behaviour towards kin and non-kin is kin discrimination. Hepper (1991) points out the importance of distinguishing the two because logically, inferences drawn from results in one area may not provide information about the other. Thus, individuals who do not respond differentially to kin and non-kin i.e. show no kin discrimination may be unable to recognize kin, alternately they may be perfectly well able to recognize kin but do not exhibit a discrimination in this situation. Absence of kin discrimination does not necessarily imply absence of kin recognition.

The ability to recognize and discriminate kin from unrelated conspecifics has been studied in several animal taxa including mammals (e.g. Manning *et al.* 1992; Mateo & Johnston 2000) birds (e.g. Komdeur & Hatchwell 1999 and references therein) amphibians (e.g. Masters & Forester 1995; Pfennig 1999), fishes (e.g. Olsen 1992; Brown & Brown 1996a and references therein) arachnids (e.g. Grosberg & Quinn 1986), spiders (e.g. Evans 1998, 1999) Hymenoptera (e.g. Moritz & Hillesheim 1990; Gamboa *et al.* 1996) and other insects (e.g. Joseph *et al.* 1999; Loeb *et al.* 2000).

Among salmonids, kin discrimination was first observed in juvenile coho salmon (*Oncorhynchus kisutch*, Quinn & Busack 1985). Since that report other salmonids that have been shown to have the ability to discriminate kin include Arctic charr (*Salvelinus alpinus*, Olsen 1989), Atlantic salmon (*Salmo salar*, Brown & Brown 1992), rainbow

trout (*Oncorhynchus mykiss*, Brown & Brown 1992) and brook trout (*Salvelinus fontinalis*, Hiscock & Brown 2000).

Juvenile salmonids use water borne chemosensory cues to identify kin (Olsen 1987; Moore *et al.* 1994). The identity and the mode of release of chemical cues that provide information about kinship are not very well understood. Skin mucus, bile salts, amino acids, intestinal contents and urine (Moore *et al.* 1994; Courtenay *et al.* 1997; Brown & Brown 1996a and references therein) are all potent olfactory stimulants in salmonids. Olsen (1987) reported that juvenile Arctic charr are attracted to water conditioned by conspecific urine and intestinal contents. In Atlantic salmon the olfactory cells respond more strongly to urine from siblings than to urine from unrelated conspecifics (Moore *et al.* 1994). Based on experiments with anuran tadpoles (Waldman 1985) and three salmonid species (Hoglund & Astrand 1973; Courtenay *et al.* 1997; Moore *et al.* 1994) it seems clear that behavioural responses are mediated by olfaction, therefore these cues can be referred to as odours (see Courtenay *et al.* 2001).

There are four possible mechanisms proposed for kin recognition (reviewed by Alexander 1979; Holmes & Sherman 1983; Blaustein *et al.* 1987; Wilson 1987). These mechanisms are not mutually exclusive and may be used alone or in conjunction with one another. Recognition can be based on 1) spatial distribution 2) direct familiarity or prior association 3) phenotype matching and 4) recognition alleles.

An individual might recognize kin encountered within a given location. Such a location may be a home site or territory. Cues based on location frequently mediate recognition of offspring among birds, especially during the early development of young.

In many cases, parents recognize their nests or nest site rather than chicks themselves up to the time at which chicks become mobile and broods mix (Komdeur & Hatchwell 1999). It is assumed that such a mechanism may evolve when there is a high probability that individuals found within a given location will be genetically related to one another. Holmes and Sherman (1983) suggest that this mechanism obviously depends on a close and consistent correlation between genetic relatedness and spatial distribution. The problem of using this mechanism to recognize kin however, is that any conspecific found in the particular location will be treated as kin, regardless of whether or not they are genetic relatives.

If relatives predictably occur in appropriate social circumstances, recognition could occur through social learning (Alexander 1979). Thus, individuals of the same litter within the same nest or those from one clutch may learn to recognize 'familiar' individuals. Association is the usual mechanism for recognition between mother and offspring. Recognition between siblings also depends on association among juveniles of some species. In laboratory tests spiny mice (*Acomys cahirinus*) placed in an arena more frequently huddled with siblings than with unfamiliar non-siblings (Porter *et al.* 1978). When siblings were separated at birth and reared apart they behaved like non-siblings and when non-siblings were reared together, they behaved like siblings reared together (Porter *et al.* 1981). The problem of using this mechanism to recognize kin however, is that any familiar conspecific will be treated as kin, and unfamiliar siblings as non-kin, regardless of whether or not they are genetic relatives.

Phenotype matching is the process by which an individual compares a conspecific's phenotypic characteristic to a learned or genetically dictated recognition template (Wadman 1987; Wilson 1987). The individual then assesses similarities and differences between its own phenotype and unfamiliar conspecifics. When first encountering an unfamiliar conspecific it matches the unfamiliar phenotype against the template it has learned. Phenotype matching depends on a consistent correlation between phenotype similarity and genotype similarity so that detectable traits are more alike among close relatives than distantly related individuals. There is experimental evidence for self-referent phenotype matching (Wu *et al.* 1980; Waldman 1982; Hauber & Sherman 2000), which Dawkins (1982) called the 'armpit effect'. Both individual and social learning are forms of phenotype matching enabling animals to acquire kin discrimination.

Numerous studies on a variety of vertebrates have documented evidence for phenotype matching mediated kin discrimination (e.g. Blaustein & O'Hara 1981; 1982; Buckle & Greenberg 1981; Grau 1982; Porter *et al.* 1983). Kin recognition does not require direct association in salmonids and they probably use a phenotype matching mechanism (Quinn & Hara, 1986; Winberg & Olsen 1992; Brown *et al.* 1993). Juveniles have the ability to discriminate unfamiliar kin from unrelated conspecifics on first encounter. They choose kin regardless of familiarity. Quinn *et al.* (1994) showed that kin-biased behaviour is expressed also under more natural conditions. Coho salmon, *Oncorhynchus kisutch*, reared only with siblings discriminated them from non-siblings while those reared with siblings and non-siblings did not make this discrimination (Quinn

& Hara 1986). Arctic charr, *Salvelinus fontinalis* reared in isolation did not discriminate kin from non-kin while those reared with kin did (Winberg & Olsen 1992). Brown *et al.* (1993) found that juvenile rainbow trout could not discriminate between familiar kin and unfamiliar kin and suggest that kinship is learned by some form of phenotype-matching mechanism.

Hamilton (1964) hypothesized that kin discrimination might occur as a result of 'recognition alleles' genes that code for a cue or label that would be shared by kin and would also allow the recognition of kin (Tang-Martinez 2001). Blaustein (1983) suggest that results of ground squirrels (*Spermophilus beldingi*; Holmes & Sherman 1982), macaques (*Macaca nemestrina*; Wu *et al.* 1980) and anuran tadpoles (*Rana cascadae*; Blaustein & O'Hara 1981; 1982; O'Hara & Blaustein 1981) are consistent with both phenotype matching and recognition alleles explanations. The possible existence of recognition alleles has been debated and it has been concluded that they are unlikely to exist due to their necessary complexity (Holmes and Sherman, 1983; Komdeur and Hatchwell 1999). Alexander and Borgia (1978) considered such alleles would be 'outlaws' helping themselves at the expense of the rest of the genome. It would be difficult, if not impossible to empirically demonstrate the existence of recognition genes (Holmes & Sherman 1983) because it is not possible to eliminate self-learning.

Spatial distribution and direct familiarity are actually indirect means by which fitness benefits could accrue to kin. Kin are not actually recognized but those individuals most likely to be kin are the ones most likely to be aided. However, recognition errors may occur if these are the primary means of recognition. Recognition errors could also

occur if mechanism three; phenotype matching; were utilized. This could happen if individuals have a similar phenotype marker but coded by different genes, or if the same genes coded for similar markers but the individuals were unrelated. Individuals can share alleles without common descent. If the individual use fourth 'recognition allele' mechanism, unrelated individuals sharing alleles are chosen and related individual not having the allele could be rejected. However, if the matched locus is highly polymorphic, kin are most likely to be preferred because the chance of unrelated individuals carrying such similar alleles is rare in nature.

According to Grafen (1990), a definition of kin recognition is 'recognition by genetic similarity detection'. He claims that only one study on kin recognition demonstrated true recognition of kin (the tunicate study by Grosberg & Quinn 1986, see later) and most of the studies that have shown evidence for kin recognition are by-products of species, group or individual recognition. For example, animals may learn the characteristics of their species by imprinting on their mother early in life. Stuart (1991) argues, in reply to Grafen (1990), that many systems using acquired standards and involving group or individual recognition may have fitness benefits associated with that recognition that 'typically flow among kin'. Many authors (Byers & Beckoff 1991; Blaustein *et al.* 1991; Stuart 1991; Sherman *et al.* 1997) do not agree with this justification for restricting the definition of kin recognition only to that mediated by genetically specified cues. Natural selection should favour individuals that use any available information about kinship to increase their inclusive fitness, regardless of

whether this information is of genetic or environmental origin (Gamboa *et al.* 1986; Ratnieks 1990).

Each animal has an individual body odour or chemical fingerprint which is in part determined by its genes (Brown 1979; Halpin 1986). Thomas (1974) suggested that the genetic individuality provided by the major histocompatibility complex (MHC) at cellular level might influence individuality at the behavioural level. MHC genes arose early in the evolution of vertebrates in response to an increased need for protection against parasites. The MHC comprises a group of genes, some of whose members are the most polymorphic functional loci known in vertebrates (Klien 1986). Because of its key function in immune response, the MHC has been studied extensively by immunologists and is consequently one of the best characterised genetic complexes in vertebrates. The functional role of the MHC is best known from studies on tissue transplantation. Incompatibility of MHC types causes rejection of grafts. Nearly every cell in the body carries molecular markers of individuality, or the gene products of the MHC. The large number of alleles at each of these loci provides so many different combinations that virtually no two individuals are identical in their MHC genotype (except in identical twins and highly inbred populations; see Brown and Eklund 1994). Two types of MHC genes, class I and class II, are important for cellular recognition. Each type codes for cell-surface glycoproteins that play a critical role in immune reactions. Class I molecules are expressed on every nucleated cell of the body except in sperm and certain cells (e.g. neurons, early fetal cells) and class II molecules are found on certain cells of the immune system (Klein 1986).

The MHC has been shown to have a role in the production of cues used to signify genetic relatedness (e.g. Boyse *et al.* 1991) which can be used in kin recognition, inbreeding avoidance in mate choice, cooperative behaviours and induced abortion (Brown and Eklund 1994). Urine emits an odour unique to each individual, which is directly related to the MHC type (Yamazaki *et al.* 1976; Singh *et al.* 1987; Brown *et al.* 1989). The first observation of an effect of MHC genotype at the behavioural level on mate choice was reported by Yamazaki and colleagues (1976), in mice. Tests were conducted in which males of inbred mice strains were caged individually with two estrous congenic females which differed from each other only in the MHC region. Under these test conditions, mating were more frequent with females of one MHC type than with the other. These initial data established that genetic differences restricted to the MHC were somehow capable of providing the basis for discriminative behaviour. The mechanism by which genetic information at MHC is translated into unique individual odour has not well known.

Histocompatibility systems that are used at the behavioural level have been studied in a wide range of organisms from sponges, bryozoans and cnidarians to primates (Grosberg 1988). The study on protochordate allorecognition is controlled by an MHC-like genes system (Scofield *et al.* 1982). In tunicates of the genus *Botryllus*, colonies are usually clones of individuals (zooids) that have grown a common vascular network and gelatinous tunic. Colonies begin from a founder individual that metamorphoses from a swimming tadpole-like larva. The colony fusion is controlled by a single highly polymorphic genetic region, similar to the MHC of vertebrates (Grosberg & Quinn 1986).

Genes of the MHC have been studied in wide range of vertebrates (see Chapter 3). Brown and Eklund (1994) suggest that many of the needed molecular and genetic data are already on hand for the study of MHC-based kin recognition in vertebrates because of the importance of MHC in immune function. Recently, MHC based detection of genetic similarity in kin discrimination (Olsen *et al.* 1998) and mate choice (Landry *et al.* 2001) has received experimental evidence from some salmonid species which will be discussed in detail in later chapters.

Despite the large amount of work on kin discrimination, there is little evidence on its functional significance (Blaustein *et al.* 1991; Brown & Brown 1996b; Brown *et al.* 1996). There are two main reasons why it might be beneficial to animals to discriminate kin. First, as discussed above, helping relatives may enhance the indirect component of inclusive fitness (Hamilton 1964). The second benefit of kin recognition is in mate choice, optimizing the balance between inbreeding and outbreeding (Bateson 1978; Shields 1982) or increasing the heterozygote advantage (Brown 1997; Landry *et al.* 2001). Sherman *et al.* (1997) described the functions of kin discrimination in other context. Anuran tadpoles associate preferentially with siblings that smell like the natal site which provide a safe, food-rich environment (Pfennig 1990). Another function of kin recognition may be disease avoidance. Cannibalistic Arizona tiger salamander (*Ambystoma tigrinum nebulosum*) larvae avoid eating close kin (Pfennig *et al.* 1991; 1993). This may prevent infections especially transmissible among close relatives because they have a similar immune system (Pfennig *et al.* 1994). It has also been suggested that animals of similar genotypes may compete more than dissimilar ones, and

kin recognition may allow animals to avoid such competition (Barnard 1990) and results in increased growth and reduced size variation among conspecifics reared with full siblings (Brown *et al.* 1996)

The studies in this thesis were designed to examine the influence of genetic and environmental cues on kin discrimination and possible adaptive significance of the kin discrimination abilities in two salmonid species Atlantic salmon (*Salmo salar*) and brook trout (*Salvelinus fontinalis*). In Chapter 2, I investigate the effect of diet on kin discrimination in juveniles of the two species. In Chapter 3, I describe the technique of isolation and characterization of MHC class II B1 locus from Atlantic salmon and brook trout. Chapter 4 examines the effect of MHC genes on kin discrimination in the two species. Chapter 5 further examines the interaction of dietary and genetic cues on kin discrimination of juvenile Atlantic salmon. Chapter 6 was designed to examine the effects of kinship on the growth of juveniles of Atlantic salmon brook trout and the adaptive significance of kin discrimination. Finally, Chapter 7 provides a summary of the observed results of all the experiments.

Kin discrimination incorporates full siblings, half siblings, cousins, aunts/uncles (Hepper 1991). However, I use kin discrimination throughout the thesis to refer to the discrimination of full siblings. In this thesis I use kin to refer to the full siblings that share alleles by common descent and non-kin to refer to unrelated individuals that do not share a recent common ancestor. When referring to literature published in salmonids and other vertebrates I use the term siblings/kin and non-siblings/non-kin as is the term used by the authors.

CHAPTER 2
EFFECT OF DIET ON KIN DISCRIMINATION IN JUVENILE
ATLANTIC SALMON AND BROOK TROUT

2.1 Introduction

Environmental cues, those not of genetic origin, involved in kin recognition have not been studied as much as genetic cues. Hiscock & Brown (2000) demonstrated that the density of fish provide 'cues' which influence kin discrimination in brook trout where juveniles preferred higher cue concentrations. They argued that juvenile brook trout use the water concentration as an indicator of shoal size and when shoal size is equal they may prefer to shoal with kin but when non-kin form larger shoals than kin they may prefer larger shoals regardless of kinship.

Another environmental cue which has been examined in some vertebrate groups is diet. Among rodents, rat pups are able to discriminate between body odours of their mothers and those of other lactating females only when the two are maintained on different diets (Leon 1975). Furthermore, exposure to particular diets, even if confined to prenatal period, can affect later preferences. When rat pups had only prenatal experience with a particular diet, they subsequently showed a preference for that diet (Hepper 1988). Adult female spiny mice (*Acomys cahirinus*) preferred pups born to mothers maintained on the same diet to pups born to mothers on a different diet (Doane & Porter 1978). Studies have shown that dietary changes alter the urine odours of guinea pigs (Beachamp 1976), and feces odours of gerbils (Skeen & Theissen 1977), mouse (*Mus musculus*; Breen & Leshner 1977; Brown & Wisker 1989), and rats (Galef 1981). Hudson & Distel

(1995) showed that rabbits prefer the females fed with a similar diet to their mothers at birth and at weaning than the females fed with a different diet.

The role of dietary cues in providing discriminable body odours has also been studied in anurans. Results of Gamboa *et al.* (1991) and Cornell *et al.* (1989) indicate that diet affects the recognition cue of larval wood frogs, *Rana sylvatica*. Larval wood frogs displayed a significant spatial preference for odours associated with familiar food over odours associated with unfamiliar food and were able to discriminate between non-kin with whom they shared a common diet and non-kin reared on a different diet (Gamboa *et al.* 1991). In the spadefoot toad (*Scaphiopus multiplicatus*), tadpole dietary cues affected spatial proximity to conspecifics (Pfennig 1990). These tadpoles preferred unfamiliar nonsiblings reared on the same diet to unfamiliar siblings that were reared on a different diet. They preferred the cues they learned from their environment. Tadpoles of common frogs (*R. temporaria*) use genetic cues in kin recognition but prefer environmental cues when they were experimentally exposed to different diets (Waldman 1991; Hepper & Waldman 1992).

Among fishes, Bryant and Atema (1987) showed that diet manipulation changes the body odours of bullheads, *Ictalurus nebulosus*. They suggested that amino acids and other nonspecific metabolites are important parts of the body odours which carry information to other members of the social group. The influence of diet on kin discrimination has not been explored in any salmonid species. The empirical investigations of salmonid kin recognition have been done on juveniles reared under uniform environmental conditions where the only detectable difference was in gene

products. This study examines the effect of diet on the ability to discriminate kin from non-kin in juveniles of Atlantic salmon and brook trout.

2.2 Methods

2.2.1 Experimental animals

I collected eggs and sperm from males and females of laboratory held brook trout and wild caught (Trepassey, a tributary in Avalon peninsula, Newfoundland) Atlantic salmon. Kin groups were created by single-pair (1 male x 1 female) mixing. Non-kin groups were created by fertilizing the pooled eggs of four females with the pooled milt of four males according to the protocol used by Hiscock and Brown (2000). Fertilized eggs of both kin and non-kin groups were placed in separate trays in an incubator with a continuous fresh water supply (surface water from a pond close to the lab. After yolk absorption the fry were transferred into 40L tanks, one for kin and one for non-kin groups for both species. One month later they were placed in 1m cylindrical tanks (water volume 0.3 m³) kin and non-kin separately, with a continuous supply of fresh water. I initially fed the fry with salmon/trout starter feed (Vextra, crude protein 53%, crude fat 20%, moisture 8%, ash 11%, fibre 1%, and nitrogen free elements (NFE) 7%: Diet 1) until three months post-hatch. Kin and non-kin groups were then divided in to three separate kin groups and three separate non-kin groups. Then, one month prior to observations, I started feeding two kin groups and two non-kin groups with two different diets (Nutra Marine, crude protein 60%, crude fat 12%, moisture 6%, ash 11%, NFE 11%: Diet 2 and Herring, protein 65%, fat 32%, moisture 1.5%, ash 1.5%: Diet 3), while the other kin group and

non-kin group were continued to be fed with the original salmon/trout feed. I fed the fish to apparent satiation on the first day ($\sim 10\%$ mean body weight) and the same amount of feed was provided once a day for one month. Testing began approximately four months post-hatch (mean weight \pm SE 2.25 ± 0.81 g, 6.46 ± 1.91 g, mean length \pm SE 3.88 ± 0.72 cm, 6.02 ± 0.91 cm for Atlantic salmon and brook trout respectively). For each trial I used 20 test fish and each fish was tested only once.

2.2.2 Experimental procedure

I tested the fish using an opaque acrylic tank (Figure 2.1) similar to that used by Quinn and Busack (1985) and Hiscock and Brown (2000). Four treatments were run giving two choices for the test fish: 1) same diet kin versus same diet non-kin 2) same diet kin versus different diet kin 3) same diet non-kin versus different diet non-kin 4) same diet non-kin versus different diet kin. Treatment 1 had 3 trials and treatments 2 to 4 had 6 trials each.

I followed the procedure used by Hiscock & Brown (2000) and began experimental trials one month after introducing the two new diets. Fish were kept in conditioning tanks for 30min (cue water concentration 12 g/l) and this cue water was collected into 25 liter buckets. Ambient fresh water and cue water from the 25 liter buckets were fed directly into the each choice alley at approximately 2 L/minute (min) and 1 l/min respectively (total flow rate 8 cm/s). A single fish was placed in the no choice/start area of the test tank for a 5 min acclimatization period and then the flow of cue water was started. The fish was given another 10 min to acclimatize. The perforated barrier separating the start area from the choice alleys, was lifted and the movement of

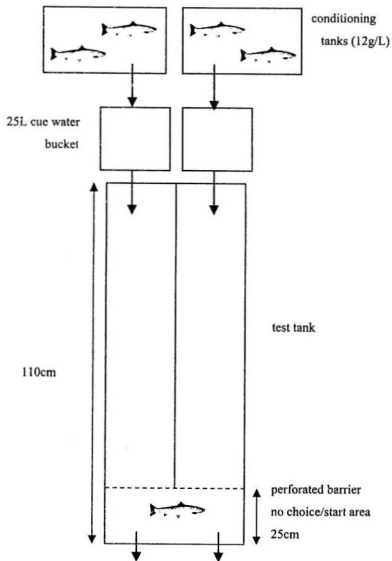


Figure 2.1 Schematic diagram of the two-choice test tank, cue water buckets and conditioning tanks.

the fish into the alleys was recorded. Observations were done for 10 min. Time spent in each alley was recorded using a Tandy 102 portable computer with 'The Observer' event recording software (Noldus 1990). The proportion of time spent in each alley was calculated by dividing the total time spent in one alley by the total time spent in both alleys and no choice area. The test fish was recorded as making a choice when half of its body had crossed the position of the removable barrier. The location of water was altered randomly in each trial to avoid location bias. The tank and the buckets were rinsed with fresh water between trials to remove any chemosensory cues remaining from the previous trial. Water temperature ranged over the study period between 13 and 20 °C. The proportion of time spent in the two choice alleys was analyzed using a Wilcoxon's matched-pairs signed-ranks test (Siegal 1988).

2.3 Results

2.3.1 Treatment 1: Same diet kin *versus* same diet non-kin

In treatment one test fish spent a significantly greater proportion of time in the alley with cue water conditioned by kin over water conditioned by non-kin for all diets (Table 2.1 & Figure 2.2). These results are consistent with those of Hiscock and Brown (2000) and Brown and Brown (1992) in which the ability to recognise kin in brook trout and Atlantic salmon has been demonstrated. The results from the three trials of treatment 1 also show that for both Atlantic salmon and brook trout, the juveniles are able to distinguish between kin and non-kin when they were reared under uniform environmental conditions

Table 2.1. Statistical comparisons of proportion of total time spent in the two choice alleys (same diet kin and same diet non-kin) by Atlantic salmon and brook trout in treatment 1.

| Diet | Atlantic salmon (Z) | Brook trout (Z) |
|-----------------------|---------------------|-----------------|
| Diet 1 (Vextra) | -3.804* | -2.503* |
| Diet 2 (Nutra marine) | -2.908* | -2.387* |
| Diet 3 (herring) | -1.982* | -2.154* |

Wilcoxon's matched-pairs signed-rank test (Z) * $P < 0.05$, ns=not significant

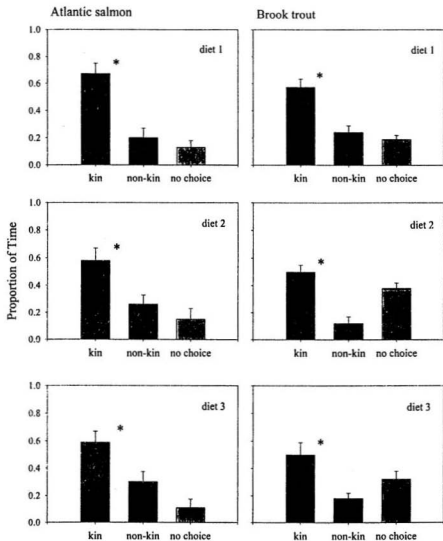


Figure 2.2 Chemosensory responses as the mean proportion of time spent in the two choice alleys and no choice area of the test tank for juveniles of Atlantic salmon and brook trout in treatment 1 (same diet kin vs same diet non-kin). Vertical bars = standard error, $n=20$ for each trial, * denotes significant differences at $p < 0.05$.

Table 2.2 Statistical comparisons of proportion of total time spent in two choice alleys (same diet kin and different diet kin) by Atlantic salmon and brook trout in treatment 2.

| Trial | Test Fish | Atlantic salmon (Z) | Brook trout (Z) |
|-----------------------------|-----------|---------------------|-----------------|
| Diet 1 <i>versus</i> Diet 2 | Diet 1 | -1.301 ns | -1.345 ns |
| | Diet 2 | -2.515* | -1.345 ns |
| Diet 1 <i>versus</i> Diet 3 | Diet 1 | -0.910 ns | -0.374 ns |
| | Diet 3 | -3.814* | -2.931* |
| Diet 2 <i>versus</i> Diet 3 | Diet 2 | -1.677 ns | -1.673 ns |
| | Diet3 | -2.881* | -3.398* |

Wilcoxon's matched-pairs signed-rank test (Z) * $P < 0.05$, ns=not significant

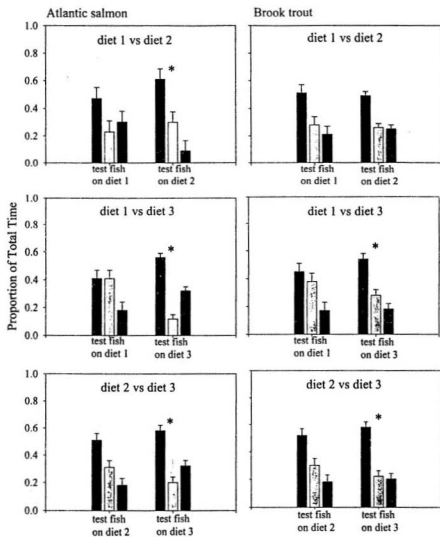


Figure 2.3 Chemosensory responses as mean proportion of time spent in the two choice alleys and no choice area of the test tank for juveniles of Atlantic salmon and brook trout in treatment 2 (same diet kin vs different diet kin). Vertical bars= standard error, $n=20$ for each trial, * denotes significant differences at $p < 0.05$.
 — same diet kin □ different diet kin — no choice

sharing a common diet.

2.3.2 Treatment 2: Same diet kin *versus* different diet kin

Juveniles in treatment two did not always show a significant preference to water conditioned by kin that shared a common diet over kin reared on a different diet (Table 2.2 & Figure 2.3). Both Atlantic salmon and brook trout test fish fed with diet 2 (Nutra marine) and diet 3 (herring) showed a significant preference for kin that shared a similar diet when tested in the diet 2 *versus* diet 3, and diet 1 *versus* diet 3 trials (Table 2.2 & Figure 2.3). When Atlantic salmon and brook trout were fed with diet 1, they did not prefer kin that shared that diet over kin fed with a different diet. Moreover, an inter-species difference in the discrimination was observed. Atlantic salmon test fish on diet 2 were able to discriminate individuals from diet 2 and diet 1. Test fish significantly preferred the cue water conditioned by donor siblings sharing a common diet over siblings that had been reared on a different diet. However, brook trout test fish on diet 2 showed no discrimination between the same test between diet 2 and diet 1.

2.3.3 Treatment 3: Same diet non-kin *versus* different diet non-kin

Results for treatment three showed a significant preference by juveniles of both species for non-kin sharing a common diet, over non-kin with a different diet (Table 2.3 & Figure 2.4). Test fish preferentially affiliated with cues associated with a common diet. They preferred cue water conditioned by non-kin that had been fed with the same diet over the non- kin fed with a different diet.

2.3.4 Treatment 4: Same diet non-kin *versus* different diet kin

Table 2.3 Statistical comparisons of proportion of total time spent in two choice alleys (same diet non-kin and different diet non-kin) by Atlantic salmon and brook trout in treatment 3.

| Trial | Test Fish | Atlantic salmon (Z) | Brook trout (Z) |
|-----------------------------|-----------|---------------------|-----------------|
| Diet 1 <i>versus</i> Diet 2 | Diet 1 | -3.014* | -2.452* |
| | Diet 2 | -4.164* | -2.966* |
| Diet 1 <i>versus</i> Diet 3 | Diet 1 | -2.242* | -1.998* |
| | Diet 3 | -3.901* | -2.084* |
| Diet 2 <i>versus</i> Diet 3 | Diet 2 | -2.036* | -2.160* |
| | Diet 3 | -3.653* | -3.986* |

Wilcoxon's matched-pairs signed-rank test (Z) * P<0.05, ns=not significant

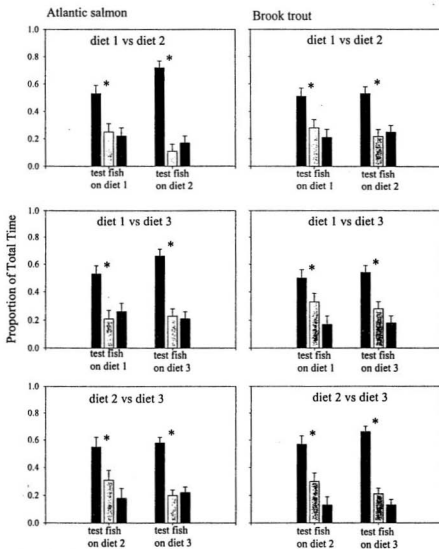


Figure 2.4 Chemosensory responses as mean proportion of time spent in the two choice alleys and no choice area of the test tank for juveniles of Atlantic salmon and brook trout in treatment 3 (same diet non-kin vs different diet non-kin). Vertical bars=standard error, n=20 for each trial, * denotes significant differences at $p < 0.05$. — same diet non-kin — different diet non-kin — no choice

Table 2.4 Statistical comparisons of proportion of time spent in two choice alleys (same diet non-kin and different diet kin) by Atlantic salmon and brook trout in treatment 4.

| Trial | Atlantic salmon (Z) | Brook trout (Z) |
|---|---------------------|-----------------|
| Diet 1 Non-kin <i>versus</i> Diet 2 Kin | -1.252 ns | -0.489 ns |
| Diet 2 Non-kin <i>versus</i> Diet 1 Kin | -1.456 ns | -0.740 ns |
| Diet 1 Non-kin <i>versus</i> Diet 3 Kin | -1.325 ns | -0.112 ns |
| Diet 3 Non-kin <i>versus</i> Diet 1 Kin | -1.307 ns | -0.636 ns |
| Diet 2 Non-kin <i>versus</i> Diet 3 Kin | -1.120 ns | -0.527 ns |
| Diet 3 Non-kin <i>versus</i> Diet 2 Kin | -0.336 ns | -0.038 ns |

Wilcoxon's matched-pairs signed-rank test (Z) * $P < 0.05$, ns= not significant

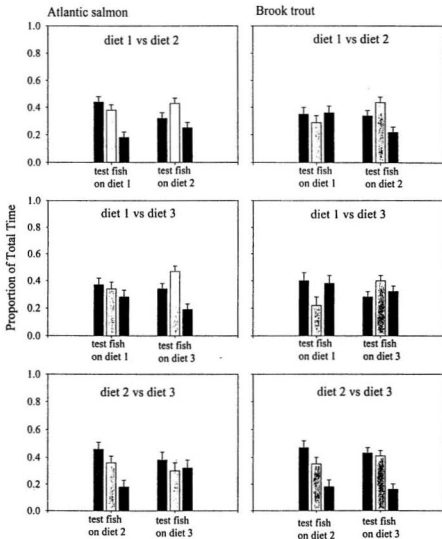


Figure 2.5 Chemosensory responses as mean proportion of time spent in the two choice alleys and no choice area of the test tank for juveniles of Atlantic salmon and brook trout in treatment 4 (different diet kin vs same diet non-kin). Vertical bars = standard error, n=20 for each trial, * denotes significant differences at $p < 0.05$.
 — different diet kin — same diet non-kin — no choice

In the fourth treatment when dietary similarity was opposite to kinship, none of the test fish showed a significant preference for either non-kin sharing a common diet or to the kin fed with a different diet (Table 2.4 and Figure 2.5). Test fish failed to discriminate kin from non-kin when kin were fed with a different diet and non-kin with the same diet. These results suggest that when dietary cues are in opposition to relatedness, neither cue appears to dominate as no reliable preference for dietary similarity or for kinship was observed.

2.4 Discussion

The results demonstrate that diet influences kin discrimination in Atlantic salmon and brook trout. Juveniles discriminated kin and non-kin when both groups shared a common diet (treatment 1, same diet kin *versus* same diet non-kin) but did not make the same discrimination when the test fish shared a common diet with the non-kin group but not with the kin group (treatment 4, same diet non-kin *versus* different diet kin). This indicates that diet cues alter an individual's phenotypic characteristic (odour) used in kin discrimination or alter the motivation of the fish to show discrimination. It could also be that fish are attracted by the residue of eating a particular diet but not recognition and discrimination of individuals. Porter *et al.* (1989) showed that in spiny mice (*Acomys cahirinus*) dietary and genotypic components have an additive effect on recognition. I also predicted that diet and genotype would contribute additively to the recognition cues and that the test fish would show a stronger affiliation to the cue water conditioned by kin

who shared a common diet (treatment 2, same diet kin *versus* different diet kin). However, the results did not provide conclusive evidence for this. In the treatment 3 (same diet non-kin *versus* different diet non-kin) juveniles significantly preferred non-kin sharing a common diet. This indicates that in the absence of cues from kin, dietary cues may be used in the recognition process.

Phenotypic variance of an individual is the combined effect of both genetic and environmental factors (Falconer 1990). Grafen (1990) suggested that variance in phenotype matching in kin recognition is entirely attributable to variance in matching genotype. He ignored environmental variance and the phenotype-environment interaction which also contributes to phenotype variance. Blaustein *et al.* (1987) reported that the rearing environment influences differential treatment of conspecifics. The differences in phenotype can be produced among genetically identical individuals by differences in developmental environment (Byers and Beckoff 1991). My study also provides evidence that diet *alters* preferences and that the cues acquired from the environment play a significant role in preferences.

Juvenile salmonids use chemosensory cues in the urine to recognise kin (Olsen 1987; Moore *et al.* 1994). Studies have shown that kin preferences can be influenced by odour concentrations (Courtenay *et al.* 1997; Hiscock & Brown 2000). Courtenay *et al.* (1997) demonstrated in salmonids that higher odour concentrations were preferred over lower concentrations. In brook trout when odour concentrations were equal, juveniles make the correct choice in recognising related individuals but, when given a choice

between high and low odour concentrations juveniles preferred the high concentration regardless of kinship (Hiscock and Brown 2000). In my study only some juveniles preferred kin sharing a common diet over kin sharing a different diet but some did not display a preference. Lack of preference could be explained by the difference in the concentration of attractants. The three different diets used in this study contain different percentages of proteins and fat. The difference in protein, and possibly fat, may have contributed to the recognition cues, providing strong or weak signals. Diet 3 had the highest protein content and diet 3 was always chosen by the test fish in the treatment 2. Nitrogenous excretory products of fish include amino acids, ammonia and urea. These products can modulate the attractiveness of water containing conspecific odours acting as attractants or repellants (Olsen 1986a, b). Ammonia and urea concentrations present in urine could vary due to nitrogen intake. In some teleosts, the rate of ammonia and urea excretion increases rapidly in response to feed intake (Engin & Carter 2001 and references therein). The majority of excreted nitrogen is derived from deamination of amino acids from dietary proteins (Wood 1993; Brunty *et al.* 1997). Having the donor fish producing different levels of ammonia and urea concentrations due to differences in dietary protein level may make the discrimination more difficult. The test fish may be selecting the odour cues from donors that were producing higher concentrations of attractants in spite of the fact that they were fed similar diets.

Dosdat *et al.* (1996) showed that ammonia excretion patterns were related to nitrogen intake but suggested no inter-species difference. However, in the same study

they showed that urea-nitrogenous excretion rates were species specific. In the trial between same diet kin and different diet kin, an inter-species difference in the odour preference was observed. Atlantic salmon juveniles fed with diet 2 preferred same diet kin over different diet kin when tested against diet 1 but juveniles of brook trout did not make the same discrimination. This could be because the two species are producing different levels of urea during nitrogen excretion even if they have a similar food intake. It is possible that if the two species are producing different amounts of urea during excretion, and hence different cue water concentrations, it could have interfered with their discrimination ability.

Pfennig (1990) showed that the spadefoot toad (*Scaphiopus multiplicatus*) preferentially associated with unfamiliar non-kin reared on the same food over unfamiliar kin reared on a different diet. Based on his observations he suggests that tadpoles prefer the cues learned early in ontogeny, regardless of the cue's source. In my study all groups of kin and non-kin were reared on the diet 1 initially, and then were switched to diet 2 and diet 3 one month prior to the observations. Juveniles did not prefer the same diet kin over different diet kin, even though they shared a common diet (diet 1) during their early ontogeny in treatment two. If the juveniles preferred diet cues learned in early ontogeny, test fish should have always selected individuals fed diet 1. Results from my study show that early environment had no effect on the choice made by juveniles. They did not show any preference for the cues from the environment they encountered during early development. Cue water concentration may be more important for the choice they made

or recent exposure to a new diet/environment may have replaced the memory of old cues. However, a lack of preference does not necessarily mean absence of recognition. Neither does it indicate that they are incapable of learning and forming long-term memory. They may have the ability to recognize but do not demonstrate behaviourally because of other factors that seem more important at this stage. Whatever the basis for this, the species in my study did not respond to learned cues early in ontogeny as did Pfennig's (1990) tadpoles of spadefoot toads.

Diet affects metabolic by-products and hence the odours produced by juveniles, not only associated with urine but feces as well. The mechanism through which dietary factors produce odour cues has not been well documented. In rats, commensal bacteria are important in determining the unique urinary odour (Schellinck *et al.* 1992). Leon (1974) suggested that the sucrose content of the rat diet (Teklad diet) may eliminate production of cecal bacteria and thus eliminate the production of discriminable odours. Later Brown and Schellinck (1995) analysed bacteria of fecal samples from rats and found that numbers of colonies of both gram negative and gram positive bacteria were higher in the feces when they were fed with Teklad diet than when they were on a different diet (Purina). Schellinck *et al.* (1997) suggest that a change in odour resulting from quantitative and qualitative changes in bacteria is likely the basis of the odour differences in rats on these two diets.

My study shows that both genetic and diet cues are important in kin recognition. Assessing the relative importance of different cues, whether genetic or environmentally

acquired, in kin discrimination can be difficult. Some studies (Pfennig 1990; Brown *et al.* 1996; Schellinck *et al.* 1997) suggest that diet provide a more salient cue for discriminating odours than genetic-related odours. Pfennig (1990) found that both genetic and dietary cues affected spatial proximity to conspecifics in the spadefoot toad, *Scaphiopus multiplicatus*, although dietary cues overwhelmed genetic cues. Rats learn and remember dietary cues more readily than genetic cues and the diet cues may mask genetic cues (Brown *et al.* 1996; Schellink *et al.* 1997). Genetic cues are known to provide consistent cues of individuality (Haplin 1991) because they do not vary according to time and location. Schellink *et al.* (1997) suggest that environmental conditions, such as those provided by diet, may vary over time and location, and it is unlikely that a dietary factor alone could provide consistent cues for recognition. Genetic cues are more useful for organisms that live in homogenous environments. For those that occur in more diverse environments a combination of both genetic and environmental cues may contribute to the phenotypic characteristics which are matched during kin recognition.

How do I apply these laboratory findings to those of salmonids in their natural habitat? There was a considerable difference between the three diets used in this study. These contrasting diets may be more extreme than those experienced by the juveniles in the field and it is possible that these diets may have produced more salient differences, and consequently affected the fish's perception of genetic cues.

CHAPTER 3

ISOLATION AND CHARACTERIZATION OF THE MAJOR HISTOCOMPATIBILITY COMPLEX CLASS II B1 EXON FROM ATLANTIC SALMON AND BROOK TROUT

3.1 Introduction

Genes of the major histocompatibility complex (MHC) have been isolated in all the vertebrates including, mammals, birds, reptiles, amphibians and fish. In all tetrapods studied so far, the class I and II regions are closely linked (Klein 1986; Trowsdale 1995), but in teleosts the two classes are on separate chromosomes (Sato *et al.* 2000). Hashimoto *et al.* (1990) first reported the structure of MHC genes in a fish using PCR with degenerate primers from conserved regions. They isolated both class I and class II sequences from carp (*Cyprinus carpio*). This initiated several efforts to isolate MHC genes from other teleosts.

MHC genes of both class I and class II have been isolated and characterised in several salmonid species including rainbow trout (*Oncorhynchus mykiss*; Juul-Madsen *et al.* 1992), Atlantic salmon (*Salmo salar*; Grimholt *et al.* 1993; Hordvick *et al.* 1993), pink salmon (*Oncorhynchus gorbuscha*; Katagiri *et al.* 1996), chinook salmon (*Oncorhynchus tshawytscha*; Miller & Withler 1997), and Arctic char (*Salvelinus alpinus*; Olsen *et al.* 1998). In this study I examined the level of polymorphism in the MHC class II B1 exon in Atlantic salmon and brook trout in samples collected from Newfoundland. MHC polymorphism is characterized by two main features, 1) the presence of a large number of alleles at a given functional locus and 2) large numbers of

nonsynonymous substitutions between alleles. Both MHC class I and class II molecules consist of nonpolymorphic domains (class I A3, class II A2 & B2) and polymorphic peptide binding domains (class I A1 & A2; class II B1 and partly A1). A high level of polymorphism at the MHC class I and class II B gene has been documented in several populations of Atlantic salmon (Langefors *et al.* 1998; Landry & Bernatchez 2001). I amplified MHC class II exon from Atlantic salmon and brook trout using primers that were designed from cDNA sequences of Atlantic salmon (*Salmo salar*, Grimholt *et al.* 1993; Horvick *et al.* 1993). Amplified domains were analysed using denaturing gradient gel electrophoresis (DGGE; Fischer & Lerman 1983). MHC genes of brook trout have not been studied and here I report the first isolation of MHC from a brook trout.

3.2 Methods

3.2.1 Sample collection

A total of 37 Atlantic salmon and 24 brook trout were collected from four different locations in Newfoundland. Twenty Atlantic salmon and 4 brook trout samples were collected from land-locked populations in the West Salmon River near St. Alban's hydro dam in the Southeast coast of Newfoundland. Seven Atlantic salmon and 18 brook trout were from a tributary at Trepassey. Seven Atlantic salmon samples were collected from Gander and 2 brook trout from Mt. Carmel Pond. Fin clips (1 cm²) were cut and preserved in 95% ethanol. Genomic DNA was extracted and MHC class IIB1 exon was amplified using the polymerase chain reaction (PCR).

3.2.2 DNA extraction and amplification

Genomic DNA was extracted from approximately 0.1 g fin clips using the chelex method. Tissue samples were placed in 200 µl of chelex extraction buffer (0.1% Tween-20, 0.1 mg/ml proteinase K, 5% chelex resin) and incubated for 15 min @ 50°C and 15 min @ 95°C. PCR of the MHC class II B1 exon was carried out in a total volume of 50 µl containing 1 µl of extracted DNA (0.2-0.5 µg), 10 pmol of each primer, 200 µM of each dNTP, and 1.3 U of Taq polymerase (PE Biosystems, Foster City, CA, USA), and 1x PCR buffer. The PCR profile included a 3 min hotstart (at 94°C) followed by the addition of Taq at 80°C, 35 cycles of 94°C, 1 min; 51°C, 2 min; 72°C, 2 min and a final extension at 72°C for 10 min. The primers classIIB1-sense 5' TGC CGA TAC TCC TCA AAG GAC 3' and classIIB1-antisense 5' cl-ACC TGT CTT GTC CAG TAT GG 3', were derived from salmonid sequences in Hordvick *et al.* (1993) and Miller and Withler (1996). The antisense primer contained a 40 bp GC-clamp (5'-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC).

3.2.3 Identification of alleles using DGGE

The 288 base pair B1 alleles were differentiated using denaturing gradient gel electrophoresis (DGGE; Fischer & Lerman 1983; Miller *et al.* 1999). In DGGE, alleles are identified by their sequence-specific melting properties. Alleles were separated on a Bio-Rad DCode™ apparatus (Bio-Rad Laboratories, Hercules, CA). The parallel denaturing gradient gels contained a 45%-60% range of denaturants urea/formamide and 7.5% acrylamide/bis acrylamide and were prepared using a Gradient Maker™ (Bio-Rad Laboratories, Hercules, CA) according to the Bio-Rad manual. Approximately 10-20 µl of

the amplified fragments containing loading dye was dispensed into the wells in the gel. Gels were run at 60 V for 15 h in TAE buffer (40 mM/l Tris, 40 mM/l sodium acetate, 1 mM/l EDTA, pH 7.4) heated to 54⁰ C. Brook trout DNA were electrophoresed at both 54⁰ C and 56⁰ C initially and since a better separation of alleles was observed at 54⁰ C, rest of the DNA was run at 54⁰ C. Gels were stained with ethidium bromide for 15 min and photographed using a polaroid camera on a UV transilluminator.

The expected heterozygosity at the MHC class II B1 locus was determined using the formula; $1 - \sum p^2$ (p is the frequency of each allele; Olsen *et al.* 1998). The reliability of the DGGE scoring was confirmed by sequence analysis of identified alleles.

3.2.4 DNA and amino acid Sequencing

Sequencing autoradiographs were analysed according to the procedure described in Miller *et al.* (1999), and the data was kindly provided by Dr. Kristina Miller at the Department of Fisheries & Oceans, Pacific Biological Station, Nanaimo, BC.

3.3 Results

A description of the genetic polymorphism of the MHC class IIB1 exon is summarized in Table 3.1. A total of 13 alleles was identified in Atlantic salmon adults with an expected heterozygosity of 0.76 (Figure 3.1). The MHC class II B1 exon of brook trout was amplified with primers designed for Atlantic salmon. A total of 7 alleles was found in brook trout with expected heterozygosity of 0.78 (Figure 3.2). Nucleotide sequence analysis of the PCR fragments of all the 13 alleles identified in Atlantic salmon and 7 alleles in brook trout is given in Figures 3.3 and 3.4. A slight variation in migration

Table 3.1 Number of alleles and expected (H_e) and observed (H_o) heterozygosities at the MHC B1 exon in 37 Atlantic salmon and 24 brook trout adults.

| | Number of Alleles | Expected Heterozygosity (H_e) | Observed Heterozygosity (H_o) |
|-----------------|----------------------|--------------------------------------|--------------------------------------|
| Atlantic salmon | 13 | 0.76 | 0.84 |
| Brook trout | 7 | 0.78 | 0.71 |

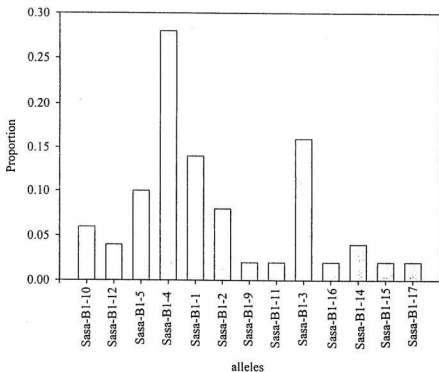


Figure 3.1 Frequency of MHC class II B1 alleles found with DGGE analyses of 37 wild caught Atlantic salmon from the West salmon river, Gander, and Trepassy.

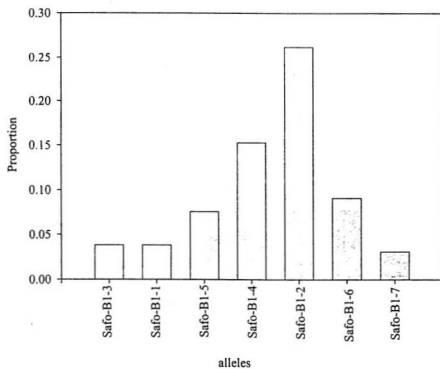


Figure 3.2 Frequency of MHC class II B1 alleles found with DGGE analyses of 24 wild caught brook trout from the West salmon river, Mt. Carmel pond and Trepassey.

| | | | | | | | | | | | | | | | | | | | | | | |
|-------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| #Sasa-B1-4 | GGT | ATA | GAG | TTT | ATA | GAC | TCT | TAT | GTT | TTC | AAT | AAG | GCT | GAA | TAT | GTC | AGA | TTC | AAC | AGC | ACT | GTG |
| #Sasa-B1-10 | ... | ... | ... | .G | .T | AC. | ..G | ... | ... | ... | ... | C.. | ... | ... | A.. | A.. | ... | ... | ... | ... | ... | ... |
| #Sasa-B1-5 | ... | ... | ... | ... | T.. | ... | ... | ... | ... | ... | ... | C.. | ... | ... | A.. | ... | ... | ... | ... | ... | ... | ... |
| #Sasa-B1-15 | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | C.. | ... | ... | A.. | ... | ... | ... | ... | ... | ... | ... |
| #Sasa-B1-14 | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | C.. | ... | ... | A.. | A.. | ... | ... | ... | ... | ... | ... |
| #Sasa-B1-1 | ... | ... | ... | ... | ... | C.. | ... | ... | ... | ... | ... | C.. | ... | ... | A.. | A.. | ... | ... | ... | ... | ... | ... |
| #Sasa-B1-2 | ... | ... | ... | ... | ... | C.. | ... | ... | ... | ... | ... | C.. | ... | ... | A.. | A.. | ... | ... | ... | ... | ... | ... |
| #Sasa-B1-3 | ... | C.. | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... |
| #Sasa-B1-12 | ... | ... | ... | .G | .T | AC. | ..G | ... | ... | ... | ... | C.. | ... | ... | A.. | ... | ... | ... | ... | ... | ... | ... |
| #Sasa-B1-11 | ... | ... | ... | .G | .T | AC. | ..G | ... | ... | ... | ... | C.. | ... | ... | A.. | ... | ... | ... | ... | ... | ... | ... |
| #Sasa-B1-16 | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | C.. | ... | ... | A.. | ... | ... | ... | ... | ... | ... | ... |
| #Sasa-B1-9 | ... | ... | ... | .G | .T | AC. | ..G | ... | ... | ... | ... | C.. | ... | ... | A.. | ... | ... | ... | ... | ... | ... | ... |
| #Sasa-B1-17 | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | C.. | ... | ... | A.. | ... | ... | ... | ... | ... | ... | ... |

| | | | | | | | | | | | | | | | | | | | | | | |
|-------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| #Sasa-B1-4 | GGG | AAG | TAT | GTT | GGA | TAC | ACT | GAG | TAT | GGA | GTG | AAG | AAT | GCA | GAA | GCC | TGG | AAC | AGT | GAT | GCT | GCG |
| #Sasa-B1-10 | ... | ... | .T. | ... | ... | ... | ... | ... | C.. | ... | ... | ... | ... | ... | ... | ... | ... | ... | AA | .G. | C.. | .A. |
| #Sasa-B1-5 | ... | ... | .T. | ... | ... | ... | ... | ... | CT. | ... | ... | ... | ... | ... | ... | ... | ... | ... | AA | .G. | C.. | .A. |
| #Sasa-B1-15 | ... | ... | .T. | ... | ... | ... | ... | ... | C.. | ... | ... | ... | ... | ... | ... | ... | ... | ... | AA | .G. | C.. | .A. |
| #Sasa-B1-14 | ... | ... | .T. | ... | ... | ... | ... | ... | CTG | ... | ... | ... | ... | ... | ... | ... | ... | ... | AA | .G. | C.. | .A. |
| #Sasa-B1-1 | ... | ... | ... | ... | ... | ... | ... | ... | CTG | ... | ... | ... | ... | ... | ... | ... | ... | ... | AA | .G. | C.. | .A. |
| #Sasa-B1-2 | ... | ... | ... | ... | ... | ... | ... | ... | CT. | ... | ... | ... | ... | ... | ... | ... | ... | ... | AA | .G. | C.. | .A. |
| #Sasa-B1-3 | ... | ... | ... | ... | ... | ... | ... | ... | CTG | ... | ... | ... | ... | ... | ... | ... | ... | ... | AA | .G. | C.. | .A. |
| #Sasa-B1-12 | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | AA | .G. | C.. | .A. |
| #Sasa-B1-11 | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | AA | .G. | C.. | .A. |
| #Sasa-B1-16 | ... | ... | .T. | ... | ... | ... | ... | ... | CTG | ... | ... | ... | ... | ... | ... | ... | ... | ... | AA | .G. | C.. | .A. |
| #Sasa-B1-9 | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | AA | .G. | C.. | .A. |
| #Sasa-B1-17 | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | ... | AA | .G. | C.. | .A. |

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| | | | | | | | | | | | | | | | | | | | | | | |
|-------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| #Sasa-B1-4 | GGG | CTG | GCT | GGA | GAG | CGA | GGG | GAG | CTG | GAG | CGT | GTC | TGT | AAG | CAT | AAC | GCT | GAT | ATC | GAC | TAC | AGC |
| #Sasa-B1-10 | --- | --- | --- | --- | --- | .T. | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | C.. | C.. | --- | --- |
| #Sasa-B1-5 | --- | --- | --- | --- | --- | .T. | --- | --- | --- | --- | --- | TA. | --- | --- | TT. | --- | --- | CC. | --- | T.. | --- | --- |
| #Sasa-B1-15 | --- | --- | --- | --- | --- | .T. | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | CC. | --- | T.. | --- | --- |
| #Sasa-B1-14 | --- | --- | --- | --- | --- | .T. | --- | --- | --- | --- | --- | T. | --- | --- | --- | --- | --- | --- | C.. | C.. | --- | --- |
| #Sasa-B1-1 | --- | --- | --- | --- | --- | .T. | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | C.. | C.. | --- | --- |
| #Sasa-B1-2 | --- | --- | --- | --- | --- | .T. | --- | --- | --- | --- | --- | .A. | --- | --- | .T. | --- | --- | CC. | --- | T.. | --- | --- |
| #Sasa-B1-3 | --- | --- | --- | --- | --- | .T. | --- | --- | --- | --- | --- | T. | --- | --- | --- | --- | --- | --- | C.. | C.. | --- | --- |
| #Sasa-B1-12 | --- | --- | --- | --- | --- | .T. | --- | --- | --- | --- | --- | TA. | --- | --- | --- | --- | --- | .C. | .A. | C.. | --- | --- |
| #Sasa-B1-11 | --- | --- | --- | --- | --- | .T. | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | CC. | --- | T.. | --- | --- |
| #Sasa-B1-16 | --- | --- | --- | --- | --- | .T. | --- | --- | --- | --- | --- | TA. | --- | --- | .T. | --- | --- | CC. | --- | --- | --- | --- |
| #Sasa-B1-9 | --- | --- | --- | --- | --- | .T. | --- | --- | --- | --- | --- | TA. | --- | --- | TT. | --- | --- | CC. | --- | T.. | --- | .T. |
| #Sasa-B1-17 | --- | --- | --- | --- | --- | A. | --- | --- | --- | --- | --- | TA. | --- | --- | TT. | --- | --- | CC. | --- | T.. | --- | --- |

```
#Sasa-B1-4 GCC ATA CTG GAC AAG ACA
#Sasa-B1-10 A . . . . .
#Sasa-B1-5 . . . . .
#Sasa-B1-15 . . . . .
#Sasa-B1-14 A . . . . .
#Sasa-B1-1 A . . . . .
#Sasa-B1-2 . . . . .
#Sasa-B1-3 A . . . . .
#Sasa-B1-12 . . . . .
#Sasa-B1-11 . . . . .
#Sasa-B1-16 . . . . .
#Sasa-B1-9 . . . . .
#Sasa-B1-17 . . . . .
```

Figure 3.3 Nucleotide sequences of 13 alleles of MHC class II B1 exon from Atlantic salmon. Dots (...) represent similar nucleotides in the two aligned sequences and dashes (---) indicate gaps introduced to improve the alignment.

```

#Safo-B1-2 GGT ATA GAG TTT ATA GAC TCT TAT GTT TTC AAT CAG GTT GAA GAT ATC AGA TTC AAC AGC ACT GTG
#Safo-B1-3 ... .. .C. ... ..
#Safo-B1-6 ... .. .G ... AC. ... .. A.C ... ..
#Safo-B1-4 ... .. .C.A ... ..
#Safo-B1-5 --- --- --- --- --- --- --- --- --- T. G.T ... ..
#Safo-B1-1 ... .. .C ... ..
#Safo-B1-7 ... .. A. ... .. A. ... .. C ... ..

```

```

#Safo-B1-2 GGG AAG TTT GTT GGA TAC ACT GAG CAT GGT GTG TAC AAT GCA GAA ACA TGG AAC AAA GGT TCT GAG
#Safo-B1-3 ... ..
#Safo-B1-6 ... .. .A. ... .. GTG ... T. A.G ... ..
#Safo-B1-4 ... .. .A. ... .. .TG ... T. A.G ... .. G. ... .. C
#Safo-B1-5 ... .. .A. ... .. T. ... A.G ... .. G. ... ..
#Safo-B1-1 ... .. .A. ... .. .TG ... T. A.G .C. ... .. .A. ... .G.
#Safo-B1-7 ... .. .A. ... .. .TG ... T. A.G .C. ... .. .A. ... .G.

```

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```
#Safo-B1-2 --- CTG GCT CAA GAG CTA GGG GAG CTG GAG CGT TAC TGC AAG CCT CAC GCT GAT ATC TAC TAC AGC
#Safo-B1-3 ... ... .. ... .. ... .. ... .. ... .. ... .. ... .. ... .. ... .. ... .. ---
#Safo-B1-6 ... .. ... .. ... .. ... .. ... .. ... .. ... .. ... .. TA. A.. ... .C. ... G.. ...
#Safo-B1-4 ... .. ... ..G. ... .. ... .. ... .. ... .. ... ..T. ... .. ... AG. ... ..G.. ...
#Safo-B1-5 ... .. ... .. ... .. ... .. ... .. ... .. ... .. ... ..T. ... .. ...A. A.. ...C. A. C.. ...
#Safo-B1-1 ATT ... .. ... .. ... .. ... .. ... .. ... .. ... ..T. ... .. ...A. A.. ...C. C.. ...
#Safo-B1-7 ATT ... .. ... .. ... .. ... .. ... .. ... .. ... ..T. ... .. ...A. A.. ...C. C.. ...

#Safo-B1-2 GCC ATA CTG GAC AAG ACA
#Safo-B1-3 --- --- --- --- --- ---
#Safo-B1-6 ... .. ... .. ... ..
#Safo-B1-4 ... .. ... .. ... ..
#Safo-B1-5 ... .. ... .. ... ..
#Safo-B1-1 --- --- --- --- --- ---
#Safo-B1-7 ... .. ... .. ... ..
```

Figure 3.4 Nucleotide sequences of 7 alleles of MHC class II B1 exon from brook trout. Dots (...) represent similar nucleotides in the two aligned sequences and dashes (---) indicate gaps introduced to improve the alignment.


```

#Sasa-B1-4  GIEFIDSYVF NKAEYVRFNS TVGKYVGYTE YGVKNAEAWN SDAAGLAGER GELERVCKHN ADIDYSAILD KT
#Sasa-B1-10 ...L.T.... .Q..NI.... ....F..... H..... --GPE...L ..... ..LH..T... ..
#Sasa-B1-5  ....Y.... .Q...I.... ....F..... L..... --GPE...L .....Y..F.. .P.Y..... ..
#Sasa-B1-15 ..... ..Q...I.... ....F..... H..... --GPE...V.L ..... ..P.Y..... ..
#Sasa-B1-14 ..... ..Q..NI.... ....F..... L..... --GPE...L .....F..... ..LH..T... ..
#Sasa-B1-1  ....H.... .Q..NI.... .... ..L..... --GPE...V.L ..... ..LH..T... ..
#Sasa-B1-2  ....H.... .Q...I.... .... ..L..... --GPE...L .....D..L.. .P.Y..... ..
#Sasa-B1-3  .T..... .. ..L..... --GPE...V.L .....F..... ..LH..T... ..
#Sasa-B1-12 ...L.T.... .Q...I.... .... ..L..... --GPE...L .....Y..... .ANH..... ..
#Sasa-B1-11 ...L.T.... .Q...I.... .... ..L..... --GPE...L ..... ..P.Y..... ..
#Sasa-B1-16 ..... ..Q...I.... ....F..... L..... --GPE...V.L .....Y..L.. .P..... ..
#Sasa-B1-9  ...L.T.... .Q...I.... .... ..L..... --GPE...V.L .....Y..F.. .P.Y..... ..
#Sasa-B1-17 ----- -Q...I.... .... ..L..... --GPE...R.L .....Y..F.. .P.Y..... ..

```

Figure 3.5 Amino acid sequences of 13 alleles of MHC class II B1 exon from Atlantic salmon. Dots (...) represent similar amino acids in the two aligned sequences and dashes (---) indicate gaps introduced to improve alignment.

```

#Safo-B1-2 GIEFIDSYVF NQVEDIRFNS TVGKFVGYTE HGVYNAETWN KGSE-LAQEL GELERYCKPH ADIYYSAILD KT
#Safo-B1-3 .....T. ....
#Safo-B1-6 ...L.T.... ...N.... ...Y.... V.LK.... ...YN.A.D.... ..
#Safo-B1-4 .....Q.... ...Y.... L.LK...A.. ...D...R.. ...F...S ...D.... ..
#Safo-B1-5 -----YV.... ...Y.... Y..K...A.. ...F...HN .ANH.... ..
#Safo-B1-1 .....K.... ...Y.... L.LKT.... .D.GI.... ...F...HN ..LH.----- --
#Safo-B1-7 .....N.... .K.... ...Y.... L.LKT.... .D.GI.... ...F...HN ..LH.... ..

```

Figure 3.6 Amino acid sequences of 7 alleles of MHC class II B1 exon from brook trout. Dots (...) represent similar amino acids in the two aligned sequences and dashes (---) indicate gaps introduced to improve the alignment.

on DGGE was observed for some alleles. When these alleles were verified by sequencing they turned out to be the same alleles having similar DNA sequences.

All the sequences that differed in nucleotide sequence also differed in amino acid sequences. Moreover, more nonsynonymous substitutions were observed more frequently than synonymous substitutions in both species.

3.4 Discussion

These results indicate that a high level of polymorphism, both at the allelic level and the amino acid level, is maintained in Atlantic salmon and brook trout populations used in this study. The brook trout class II B1 exon was able to isolate using Atlantic salmon primers. According to the nomenclature of the MHC proposed by Klein *et al.* (1990a,b), brook trout MHC can be named as *Mhc-Safo*. Both species displayed a high level of nonsynonymous substitutions at this locus.

DGGE is a rapid, sensitive method for the detection of nucleotide sequence variation which detects most single-base substitutions in amplified fragments by the differences in their melting behaviour (Fischer & Lerman 1983). This technique offers a great potential for use in population analysis of genetic markers with higher levels of sequence variation and in detecting single-base mutations in disease studies.

The MHC genes of teleosts are similar to those of mammals, the polymorphism of which is believed to be maintained both by parasite-driven selection and MHC-based mating preference (Klein *et al.* 1997). However, some vertebrate species have MHC loci that are virtually monomorphic (Nei & Hughes 1991) for various reasons. Severe

population bottlenecks can reduce the variation at all loci including the MHC (e.g. cheetah, *Acinonyx jubatus*, O'Brien *et al.* 1985; Syrian hamster, McGuire *et al.* 1985; mice populations found in small islands in the North Sea, Figueroa *et al.* 1986). Some vertebrates show low levels of MHC polymorphism due to low selection pressure (e.g. Syrian hamster, McGuire *et al.* 1985; fin whale, *Balaenoptera physalus*, Trowsdale *et al.* 1989; Southern elephant seal, *Balaenoptera borealis*, Slade 1992).

High polymorphism and the central role in the immune response make MHC genes highly suitable as markers in population and disease studies. Salmonids include some of the most important species in aquaculture. Domestication has resulted in a number of weaknesses mainly related to infectious diseases due to intensive selective breeding and drastic changes in the environment. Studies of MHC genes give information about polymorphism and possible changes in variability as a result of selective breeding and disease resistance or susceptibility associated with specific alleles or haplotypes.

MHC molecules are also known to mediate olfactory-based kin recognition (Yamazaki *et al.* 1976; Olsen *et al.* 1998; also see Chapter 1) and may function in mammalian mate choice as an inbreeding avoidance mechanism (Potts *et al.* 1994) or to increase the heterozygosity in offspring (Landry *et al.* 2001). I used this technique to genotype the kin and non-kin groups from Atlantic salmon and brook trout to examine whether the kin discrimination ability of juvenile Atlantic salmon and brook trout is influenced by the MHC class IIB1 exon (Chapter 4). Similarly in Chapter 5 using the same technique I investigated the possible interaction of dietary and MHC based genetic cues in kin recognition in juvenile Atlantic salmon.

CHAPTER 4

MHC AND KIN DISCRIMINATION IN ATLANTIC SALMON

AND BROOK TROUT

4.1 Introduction

Hamilton's (1964) genetical kinship theory predicts that genetic relatedness will be an important variable in the evolution of social behaviour, i.e. organisms will favour kin with whom they share alleles. Individuals most likely to share a given allele are close relatives who share alleles by common descent. The major histocompatibility complex (MHC) has been shown to influence body odour and is among the potential candidates for the genetic basis of kin recognition in vertebrates (reviewed in Brown & Eklund 1994). MHC mediated olfactory-based kin recognition was first studied in mice (Yamazaki *et al.* 1976) and later confirmed in rats (Brown *et al.* 1987). Recent studies also suggest that humans can discriminate between the odours of conspecifics with disparate MHC (Wedekind *et al.* 1995). Direct evidence that MHC influences odour came from a study using an "e-nose" that electronically detected differences in urinary odours of congenic mice differing only in their MHC alleles (Montag *et al.* 2001). Thus, the ability to discriminate odours based on MHC haplotypes in a variety of species suggests that MHC might function in social behaviour, as originally proposed in 1974 by Thomas.

Olsen *et al.* (1998) provided the first evidence that kin recognition in fish is influenced by MHC gene haplotypes. Through fluvium tests on juvenile Arctic charr (*Salvelinus alpinus*) they found that fish preferred MHC identical siblings to siblings with

different MHC genotypes and MHC different siblings were preferred over MHC different non-siblings. However, they observed no discrimination when the test fish shared one allele with non-sibling but no alleles with the sibling donor. Olsen *et al.* (1998) further predicted that adult Arctic charr similarly use MHC-based mate choice as a mechanism to avoid inbreeding. Recently, Landry *et al.* (2001) studied whether mate choice of wild Atlantic salmon is dependent on the similarity of MHC class II B genes between mates. They found that Atlantic salmon chose their mates in order to increase the heterozygosity of their offspring at the MHC but not as a mechanism of inbreeding avoidance. Further, they found that individuals could discriminate the degree of divergence among MHC alleles, and chose to mate with individuals that contained alleles with maximal divergence from their own MHC alleles. Their study provides the first evidence that MHC genes influence mate choice in fish.

The main objective of this study was to test the hypothesis that the juveniles of Atlantic salmon and brook trout can discriminate between water scented by individuals that share MHC alleles and individuals that do not share alleles. I analysed the highly polymorphic peptide-binding region of the MHC class II gene using polymerase chain reaction (PCR) in combination with denaturing gradient gel electrophoresis (DGGE) to study the genetic variation at the B1 locus. I used the information about the MHC genotype of kin and non-kin groups to study their discrimination abilities, and ascertained additional cues from the rest of the genome affect the kin recognition in the two species as well.

4.2 Methods

4.2.1 Test fish

Eggs and sperm were collected from males and females of laboratory held brook trout and wild caught Atlantic salmon. Kin groups were created by single-pair mixing. Non-kin groups were created by fertilizing the pooled eggs of four females with the pooled milt of four males according to the protocol used by Hiscock and Brown (2000). After one hour post fertilization hydration, eggs from kin and non-kin groups were placed in separate trays in one incubator with a continuous fresh water supply. After yolk absorption the fry were transferred into 40 l tanks and one month later into 1 m cylindrical tanks (water volume 0.3 m^3) with a continuous supply of fresh water. Two kin groups and one non-kin group were reared separate tanks. Fry were fed with salmon-trout starter feed (Vextra). At eight months post hatch 35 fish from each kin and non-kin group were tagged and a small piece of tail fin (1 cm^2) was cut and preserved in 95% ethanol. Tagged fish were transferred back into the tank until use for observations. I extracted DNA from the fin clips and MHC class II B1 locus was amplified in both species. Behaviour observations began approximately nine months post-hatch (mean weight $4.15 \pm 0.27 \text{ g}$, $9.86 \pm 0.40 \text{ g}$ and mean length $6.05 \pm 0.11 \text{ cm}$, $11.53 \pm 0.33 \text{ cm}$ for Atlantic salmon and brook trout respectively).

4.2.2 DNA extraction and amplification (see Chapter 3.2.2)

4.2.3 Identification of alleles using DGGE (see Chapter 3.2.3)

4.2.4 DNA and amino acid sequences (see Chapter 3.2.4)

4.2.5 Observation procedure

Fish were tested using the opaque acrylic test tank (Figure 2.1). Six trials were run: 1) kin sharing both alleles vs non-kin sharing no alleles, 2) kin sharing both alleles vs kin sharing no alleles, 3) non-kin sharing both alleles vs non-kin sharing no alleles, 4) kin sharing no alleles vs non-kin sharing no alleles, 5) kin sharing no alleles vs non-kin sharing one allele, 6) kin sharing no alleles vs non-kin sharing both alleles.

Each trial had 15-20 observations. Two donor fish with known alleles were selected, weighed and placed in 25 l buckets. Water was conditioned according to the weight of the fish (12 g/l for 30 min). A test fish with known alleles was selected from the kin group to meet the required allele combinations of each trial. A single fish was placed in the no choice/start area of the test tank for a 5 min acclimatization period. Cue water from 25 l buckets was connected into the tank and ambient fresh water and cue water were fed directly into each choice alley at approximately 2 l/min and 1 l/min respectively (total flow rate 8 cm/s). Once the flow of cue water was started the fish was given another 10 min to acclimatize. The trial began when the perforated barrier was lifted, and the movement of the fish over a 10 min period was recorded. Time spent in each alley was recorded as explained in Chapter 2. The proportion of total time spent in each choice alley was calculated by dividing the total time spent in all three areas of the test tank. The test fish was recorded as making a choice when half of its body crossed the position of the removable barrier. For each trial different test fish were used. Test fish were used once only while some of the donors were used more than once. The location of water was randomly altered in each trial to avoid location bias. The tank and the buckets were rinsed with fresh water between trials to remove any chemosensory cues remaining from the previous trial. Water temperature ranged over the

study period between 13 and 20°C. The significance of the proportions of time were analyzed using a Wilcoxon's matched-pairs signed-ranks test (Siegal 1988).

4.3 Results

4.3.1 MHC polymorphism

The Atlantic salmon parents used to create kin group were both heterozygous and had no alleles in common. The progeny consisted of four heterozygous genotypes. The brook trout parents used to create kin group were both heterozygous and shared one allele. The kin group had four genotypes, one homozygous and three heterozygous. Kin groups for both species had siblings sharing both alleles, sharing one allele and sharing no alleles. The alleles segregated independently following a Mendelian pattern of inheritance. Parents used to create non-kin groups (four females and four males) consisted of both heterozygous and homozygous males and females and shared some alleles with the parents of the kin group. This allowed me to test the individuals from the non-kin group that shared one or both alleles with the kin group.

4.3.2 Kin recognition and MHC

The first trial was a test of kin sharing both alleles and non-kin sharing no alleles, and served as a positive control for kin recognition in general (Table 4.1, Figure 4.1 & Figure 4.2). Juveniles significantly showed preference for kin sharing alleles over non-kin sharing no alleles ($p < 0.001$). Trials two and three were designed to investigate the influence of MHC on kin recognition. In the second trial juveniles showed a preference for kin sharing both alleles to kin sharing no alleles (Table 4.1, Figure 4.1 & Figure 4.2).

Table 4.1 Statistical comparisons using Wilcoxon's matched-pairs signed ranks test (Z) for the proportion of total time spent in two choice alleys of the test tank by test fish for Atlantic salmon and brook trout.

| Trial | Atlantic salmon Z | Brook trout Z |
|--|----------------------|------------------|
| 1. Kin sharing both alleles vs Non-kin sharing no alleles | -4.091 ** (n=16) | -3.661** (n=20) |
| 2. Kin sharing both alleles vs Kin sharing no alleles | -1.985 * (n=20) | -2.016 * (n=20) |
| 3. Non-kin sharing both alleles vs Non-kin sharing no alleles | -3.183 * (n=15) | -2.427 * (n=20) |
| 4. Kin sharing no alleles vs Non-kin sharing no alleles | -2.619 * (n=16) | -2.904 * (n=20) |
| 5. Kin sharing no alleles vs Non-kin sharing one allele | -0.995 ns (n=18) | -1.344 ns (n=20) |
| 6. Kin sharing no alleles vs Non-kin sharing both alleles | -0.461 ns (n=21) | -0.112 ns (n=20) |

** denotes significant differences at $p < 0.001$, and * = $p < 0.05$, ns = $p > 0.05$ (not significant)

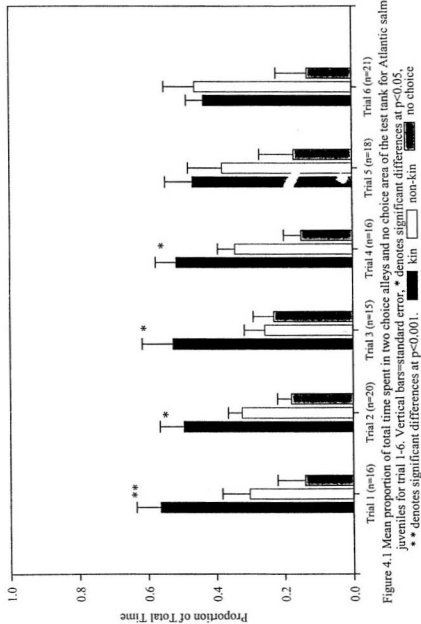


Figure 4.1 Mean proportion of total time spent in two choice alleys and no choice area of the test tank for Atlantic salmon juveniles for trial 1-6. Vertical bars=standard error, * denotes significant differences at $p<0.05$, ** denotes significant differences at $p<0.001$. kin non-kin no choice

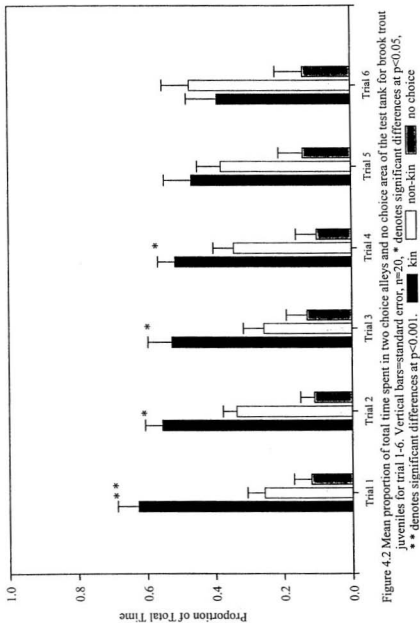


Figure 4.2 Mean proportion of total time spent in two choice alleys and no choice area of the test tank for brook trout juveniles for trial 1-6. Vertical bars=standard error, $n=20$, * denotes significant differences at $p<0.05$, ** denotes significant differences at $p<0.001$. kin non-kin no choice

However, although the preference was significant for brook trout ($p=0.008$), it was not significant at $p<0.05$ in Atlantic salmon. ($p=0.056$). Trial three presented a choice between non-kin sharing both alleles and non-kin sharing no alleles. In both species, juveniles significantly preferred non-kin sharing both alleles ($p<0.05$; Table 4.1, Figure 4.1 and Figure 4.2). The preference shown in the kin and non-kin trials for individuals sharing both MHC alleles demonstrates that MHC significantly influenced kin discrimination in both species.

The fourth trial, kin and non-kin both shared no alleles, tested whether the rest of the genome has an effect on kin discrimination. Juveniles still preferred kin over non-kin, even when they did not share any MHC alleles ($p<0.05$, Table 4.1, Figure 4.1 & figure 4.2), which suggests that kin recognition is not simply controlled by the MHC class II gene, but by a combination of genes.

Finally, in the fifth and sixth trials (kin share no alleles vs non-kin share one allele and kin share no alleles vs non-kin share both alleles) I tested the importance of the genes of the MHC relative to the rest of the genome during discrimination. No significant preference for kin sharing no alleles versus non-kin sharing either one allele or both alleles was observed ($p>0.05$, Table 4.1, Figure 4.1 & Figure 4.2).

4.4 Discussion

Grafen (1990) asked the question regarding kin recognition 'should we expect individuals to behave according to the extent of genetic similarity at the matched locus or should we expect them to behave according to the extent of genetic similarity through the

genome as a whole?’ I addressed both of these possibilities in this study, using MHC class II genes as the matched locus. My results demonstrate that genetic similarity at the MHC class II gene was used as the basis for kin discrimination among juveniles of both Atlantic salmon and brook trout. Juveniles choosing kin or non-kin sharing both MHC alleles demonstrated the significant influence of MHC on kin discrimination in both species. However, the ability to discriminate between kin and non-kin that did not share any MHC alleles reveals that additional genes were involved in kin discrimination process. Thus this study provides evidence that single salmonid MHC class II gene found in salmon is one of a number of genes involved in producing cues used in kin discrimination in at least two salmonid species, Atlantic salmon and brook trout.

I also determined the relative importance of the MHC in kin discrimination compared to the rest of the genome using unrelated individuals carrying identical MHC and a related individual carrying different MHC. Test fish did not show any preference for kin sharing no alleles when tested against non-kin sharing either one allele or both alleles. The fact that the juveniles could not differentiate kin with different MHC and unrelated individual with similar MHC indicated that the MHC is as important as the rest of the genome in kin recognition. In mice, genes on the x and y chromosomes were found to contribute to genotypic determination of odours, but were less influential than the genes of the MHC (Yamazaki *et al.* 1986).

Previous studies have shown that juvenile Atlantic salmon and brook trout can discriminate between kin and non-kin (Brown & Brown, 1992; Hiscock & Brown 2000). These studies used cue water from conditioning tanks containing several donor fish from

either kin or non-kin groups. There is a possibility that donors from both kin and non-kin groups shared alleles with the test fish in those studies. In this study I used a single donor fish with a known genotype to create cue water and observed a highly significant ($p < 0.001$) preference for kin sharing alleles over non-kin sharing no alleles. In a study by Olsen *et al.* (1998), both sibling and non-sibling groups were created by single pair mixing and the non-kin group shared one allele with the kin group. I used pooled eggs and sperm of four males and four females which allowed me to test non-kin that shared both alleles with kin that did not share any alleles and to determine the relative importance of cues from MHC versus those from rest of the genome.

Only the alleles that differ at the level of amino acid sequence affect the phenotypic odours. Yamazaki *et al.* (1990) showed that alleles differing by only a single amino acid can be discriminated. In the same study they showed that not all alleles differing in amino acid sequence present a discriminable odour difference (Yamazaki *et al.* 1990). The MHC based kin discrimination (Olsen *et al.* 1998) and most of the MHC based mating preference studies (Hedrick 1992; Paterson & Pemberton 1997) considered only the genotypic differences between individuals, and assumed that the genotypic differences are expressed in the phenotype of the animals. However, if differences among alleles are all synonymous, MHC alleles could be different at the molecular level, but they could still code for the same amino acids. In this study I determined the amino acid differences of the kin and non-kin groups that were used to test the influence of MHC on kin discrimination ability. All the different alleles in kin and non-kin groups of both

species coded for different amino acid sequences and hence the peptides produced are different.

The underlying mechanism through which MHC genes influence odour remains unclear. Five hypothesis have been proposed to explain the mechanism how MHC genes control odour (reviewed in Penn and Potts 1998). The MHC molecule hypothesis (Singh *et al.* 1987; 1988; Roser *et al.* 1991) suggests that because MHC molecules occur in urine and sweat, MHC molecules or fragments are the odourants. Second hypothesis (the peptide hypothesis; Singer *et al.* 1997) suggests that the unique pool of peptides bound by MHC molecules may be the precursors for the volatile odorants. Thirdly, microflora hypothesis, suggested by Howard (1977), assume that MHC genes may influence odour indirectly by shaping an individual's particular population of commensal microflora. The fourth hypothesis known as the carrier hypothesis suggests that MHC molecules are converted during degradation from peptide-presenting molecules into transporters that bind to aromatic molecules produced by commensal gut microbes (Pearse-Pratt *et al.* 1992). The fifth hypothesis, (peptide-microflora hypothesis) combine both peptide and microflora hypotheses in which MHC molecules alter the available pool of peptides and their metabolic products are made volatile by commensal microflora. Penn and Potts (1998) suggest that the peptide microflora hypothesis is the most consistent with available data and may help explain the disparity among different studies.

Both MHC class I and class II loci can produce differences in individual odours (reviewed in Brown & Eklund 1994). In this study, I investigated only the exon containing the peptide-binding region of the MHC class II gene. As class I and II genes

are not linked in teleost fishes (Sato *et al.* 2000) it is important to study both loci to determine whether both class I and II loci influence kin discrimination, and if so, whether they influence it individually or in combination. Since salmonid MHC has been widely studied, much of the data needed for molecular and genetic basis of kin recognition are available in these species (Miller and Withler 1996; Miller and Withler 1998; Shum *et al.* 2001).

In order to demonstrate experimentally that discrimination was made on the basis of MHC based signals, it is important to eliminate the possibility that the signals used in the discrimination process were produced by other parts of the genome. This can be achieved by creating a pair of inbred (congenic) strains that differ only at the MHC. This involves crossing a homozygous inbred strain with another strain carrying a different MHC genotype and then back-crossing to the original strain for 20 or more generations while preserving the new MHC haplotype. Such crosses have been conducted in mice that were used in studies to determine the influence of MHC on mate choice. This type of experiment is possible with mice and other rodents that have short generation times but could not be easily achieved for seasonal breeders like salmonids. And although inbred strains may be useful for controlling the genotype, the relevance of MHC-dependent kin discrimination and MHC disparate mating preferences on these strains to natural populations in the wild are difficult to extrapolate. Olsen *et al.* (1998) controlled the overall genetic similarity in their experiment using Arctic charr full siblings where the probability of sharing any allele among full siblings is 0.5. I also used full siblings in the kin group.

Hamilton (1964) suggested that kin discrimination might occur as a result of 'recognition alleles' and that individuals may favour conspecifics sharing alleles regardless of the overall genetic relatedness of those conspecifics. Thus, kin lacking the allele are placed at a competitive disadvantage to non-kin possessing the allele (see Waldman 1987). The settling of tunicate larvae with histocompatible individuals (Grosberg & Quinn 1986) is a well documented example of kin recognition using a recognition allele mechanism. In a review on kin recognition, Grafen (1990) rejected most empirical data of kin recognition and suggested that only the tunicate data demonstrate true kin recognition. However, in my study juveniles did not prefer the non-kin sharing alleles over kin sharing no alleles. Phenotype matching, which combines the overall genetic relatedness and/or the particular gene(s) together with the rearing environment, seems to be the favoured mechanism for kin recognition in salmonids (Winberg & Olsen 1992; Brown *et al.* 1993). Although this study did not directly address the phenotype matching mechanism of kin recognition, the results allow me to eliminate a recognition allele, at least for MHC, as a mechanism of kin recognition in the two salmonid species I studied.

In summary, the experimental data from the present study provides evidence that kin discrimination in Atlantic salmon and brook trout is influenced by the MHC. The salmonid juveniles compared MHC-coded cues and used the information for recognition. A similar data set was presented for Arctic charr (Olsen *et al.* 1998); hence three of the salmonid species have demonstrated MHC-based kin discrimination. It is likely that

MHC-based kin recognition will be found throughout the salmonid species, but its generality among other vertebrates awaits experimentation.

CHAPTER 5
RECOGNITION ERRORS: EFFECT OF OVERLAPPING GENETIC AND DIETARY
CUES IN KIN DISCRIMINATION IN JUVENILE
ATLANTIC SALMON

5.1 Introduction

Hamilton's (1964) kin selection theory predicts that 'the ability to influence the transmission of one's alleles onto future generations would be enhanced in organisms who can readily distinguish between related and unrelated conspecifics'. According to this theory selection should favour individuals who can recognise their relatives and make discrimination without error. Animals display remarkable success and surprising failures in discrimination among related and unrelated conspecifics. From an evolutionary point of view the failure to recognize kin is more difficult to explain than the successes (Beecher 1991 and references therein). In nature related and unrelated individuals often express overlapping cues (e.g. rodents, Lacy & Sherman 1983; and honey bees, Getz 1991) and unrelated individuals may be recognised by either mimicking the cues of kin or by scrambling cues to prevent discrimination (Reeve 1997). Reeve (1989) described two types of errors in the recognition system. These errors can be either accepting an unrelated conspecific as kin, acceptance errors, or rejecting a related conspecific as a non-kin, rejection errors.

Cues used in kin discrimination can be of genetic and/or environmental origin (Hepper 1991; also see Chapter 2 and Chapter 4). Lacy and Sherman (1983) argue that consistent cues of genetic relatedness between kin and non-kin must be provided in order

to discriminate kin by phenotype matching. The genotype provides consistent cues, but environmental cues vary according to time and location. It is important to maintain the uniqueness of genetic cues regardless of changes in environment in order to make a choice based on kinship. In previous studies I have shown that diet (Chapter 2) and MHC genes (Chapter 4) influence kin discrimination in juvenile Atlantic salmon and brook trout. Hepper and Cleland (1999) suggest that the role of the MHC in influencing kin discrimination must be sensitive to environmental factors and it is the interaction between the MHC and environmental factors that determine the exact nature of the behaviour exhibited by individuals. This study was designed to examine whether the MHC based kin discrimination is influenced by the change in diet and whether the overlapping cues cause recognition errors in Atlantic salmon.

5.2 Methods

5.2.1 Test fish

I collected eggs and sperm from wild caught females and males of Atlantic salmon. Two kin groups and one non-kin group were created and the eggs were incubated as explained in Chapter 2. Kin and non-kin groups were reared separately. After yolk absorption, the fry were transferred into 40 l tanks and one month later into 1m cylindrical tanks (water volume 0.3 m^3) with a continuous supply of fresh water. I fed the fry with salmon/trout starter feed (Vextra, [diet 1](#); protein 53%, fat 20%). After three months post hatch kin and non-kin groups were divided into two separate tanks and one

group fed with a different diet, Nutra marine (diet 2; protein 60%, fat 12%). The other two groups continued to be fed with the original diet.

At eight months post hatch, 35 fish from one kin group and non-kin group and 18 fish from the other kin group were tagged and a small piece of tail fin (1 cm^2) was cut and preserved in 95% ethanol (same sample of fish as in Chapter 4 were used in this experiment). Tagged fish were transferred back into the tank until used for observations. I extracted DNA from the fin clips and the MHC class II B1 locus was amplified in both species. Observational experiments began approximately eleven months post-hatch (mean weight $5.75 \pm 0.37\text{ g}$, and mean length $7.23 \pm 0.38\text{ cm}$).

5.2.2. DNA extraction and amplification (see Chapter 3.2.2)

5.2.3 Identification of alleles using DGGE (see Chapter 3.2.3)

5.2.4 DNA and amino acid sequences (see Chapter 3.2.4)

5.2.5 Observation procedure

I tested the fish using the same test tank as in Chapter 2 (Figure 2.1). Three experiments were performed to investigate the interaction of cues associated with diet and MHC. A third experiment investigated the effect of overlapping cues on kin discrimination. Experiment 1) discrimination between two kin groups: (Trial 1.1 same diet kin vs different diet kin; Trial 1.2. kin share both alleles/ same diet vs kin share no alleles/ same diet; Trial 1.3. kin share no alleles/ same diet vs kin share both alleles/ different diet). Experiment 2) discrimination between two non-kin groups (Trial 2.1. same diet non-kin vs different diet non-kin; Trial 2.2. non-kin share both alleles/ same diet vs non-kin share no alleles/ same diet; Trial 2.3. non-kin share no alleles/ same diet

vs non-kin share both alleles/ different diet). Experiment 3) discrimination between kin and non-kin (Trial 3.1. different diet kin vs same diet non-kin; Trial 3.2. kin share no allele/ same diet vs non-kin share both alleles/ same diet; Trial 3.3. kin share no alleles/ different diet vs non-kin share both alleles/ same diet).

The same observational and data recording procedure was used as outlined in Chapter 4.2.4.

5.3 Results

5.3.1 MHC polymorphism

Genotypes of the kin and non-kin groups were the same as in Chapter 4.3.1.

5.3.2 Experiment 1: Discrimination between two kin groups

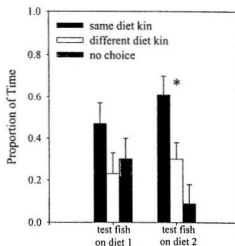
In trial 1.1 (same diet kin vs different diet kin), juveniles fed with diet 2 significantly favoured siblings fed that diet over siblings fed a different diet. However, juveniles fed with diet 1 did not show a preference for either sibling group (Table 5.1 and Figure 5.1). In trial 1.2 when both kin groups were fed with the same diet, preference for siblings sharing alleles over siblings that did not share any alleles was not significant (kin share both alleles vs kin share no alleles; Table 5.1 and Figure 5.1). When dietary similarity was opposite to allele sharing (trial 1.3, kin share no alleles /same diet vs kin share both alleles/ different diet), diet cues were preferred. Kin sharing a common diet but no alleles were preferred over kin sharing both alleles reared on a different diet when the test fish had been fed with diet 2 but they did not show a significant preference when the test fish was reared on diet 1 (Table 5.1 and Figure 5.1).

Table 5.1. Statistical comparisons using Wilcoxon's matched-pairs signed rank test (Z) for the proportion of total time spent in two choice alleys of the test tank by the test fish to the odours of different kin groups.

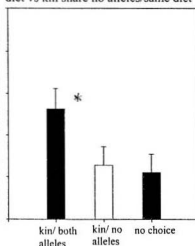
| Trail | Test Fish | Z |
|---|-----------|-----------------|
| 1. Same diet kin vs different diet kin | Diet 1 | -1.301ns (n=20) |
| | Diet 2 | -2.515 * (n=20) |
| 2. Kin share both alleles/ same diet vs kin share no alleles/ same diet | Diet 1 | -1.985 * (n=20) |
| | Diet 2 | -2.949 * (n=20) |
| 3. Kin share no allele/ same diet vs kin share both alleles/ different diet | Diet 1 | -1.437ns (n=20) |
| | Diet 2 | -2.949 * (n=20) |

* denotes significant differences at $P < 0.05$, ns = $P > 0.05$ (not significant)

Trial 1.1 same diet kin vs different diet kin



Trial 1.2 kin share both alleles/same diet vs kin share no alleles/same diet



Trial 1.3 kin share no alleles/ same diet vs kin share both alleles/ different diet

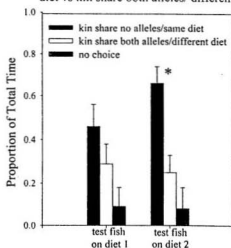


Figure 5.1 Proportion of total time spent in choice alleys and no choice area by test fish in the three trial in Experiment 1. Vertical bars = standard error, * denotes significant differences at $p < 0.05$.

5.3.2 Experiment 2: Discrimination between two non-kin groups

When the juveniles were given a choice between two non-kin groups (same diet non-kin share vs different diet non-kin), in trial 2.1, test fish significantly preferred non-kin sharing a common diet over non-kin reared on a different diet (Table 5.2 and Figure 5.2). When genotypes were tested in trial 2.2 (non-kin share both alleles/ same diet vs non-kin share no alleles/ same diet) non-kin sharing both alleles were chosen over non-kin that did not share any alleles (Table 5.2 and Figure 5.2). However when dietary and genetic cues were opposite to each other, trial 2.3 (non-kin share no alleles/ same diet vs non-kin share both alleles/ different diet), test fish did not display any significant preference (Table 5.2 and Figure 5.2).

5.3.3 Experiment 3: Discrimination between kin and non-kin groups

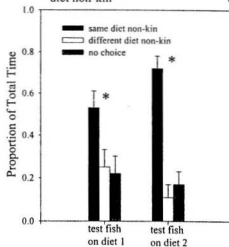
When kin were fed with a different diet and non-kin fed with the same diet, trial 3.1, juveniles did not prefer either group (different diet kin vs same diet non-kin; Table 5.3 and Figure 5.3). Similarly when kin did not share any alleles and non-kin shared both alleles, trial 3.2 juveniles did not show a preference for any group (kin share no allele/ same diet vs non-kin share both alleles/ same diet; Table 5.3 and Figure 5.3). However, test fish preferred non-kin sharing both alleles reared on a common diet over kin sharing no alleles and fed a different diet (trial 3.3). When both factors, diet and alleles were in opposition to kinship, unrelated individuals were favoured over related ones (Table 5.3 and Figure 5.3).

Table 5.2. Statistical comparisons using Wilcoxon's matched-pairs signed ranks test (Z) for the proportion of total time spent in two choice alleys of the test tank by the test fish to the odours of different non-kin groups.

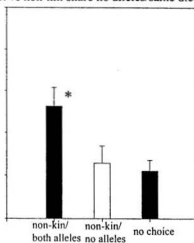
| Trail | Test Fish | Z |
|--|-----------|-----------------|
| 1. Same diet non-kin vs different diet non-kin | Diet 1 | -3.101* (n=20) |
| | Diet 2 | -4.142* (n=20) |
| 2. Non-kin share both allele/ same diet vs non-kin share no alleles/ same diet | Diet 1 | -2.613* (n=15) |
| 3. Non-kin share no alleles/ same diet vs non-kin share both alleles/ different diet | Diet 1 | -.0384ns (n=15) |
| | Diet 2 | -0.527ns (n=16) |

* denotes significant differences at $P < 0.05$, ns = $P > 0.05$ (not significant)

Trial 2.1 same diet non-kin vs different diet non-kin



Trial 2.2 non-kin share both alleles/same diet vs non-kin share no alleles/same diet



Trial 2.3 non-kin share no alleles/same diet vs non-kin share both alleles/different diet

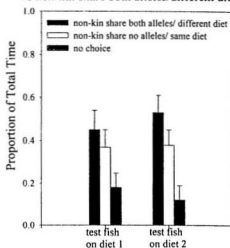


Figure 5.2 Proportion of total time spent in choice alleys and no choice area by the test fish in three trials in Experiment 1. Vertical bars = standard error, * denotes significant differences at $p < 0.05$.

Table 5.3. Statistical comparisons using Wilcoxon's matched-pairs signed ranks test (Z) for the proportion of total time spent in two choice alleys of the test tank by the test fish to the odours of different kin and non-kin groups.

| Trail | Test Fish | Z |
|---|-----------|-----------------|
| 1. Different diet kin vs same diet non-kin | Diet 1 | -1.252ns (n=20) |
| | Diet 2 | -1.456ns (n=20) |
| 2. Kin share no alleles/ same diet vs non-kin share both alleles/ same diet | Diet 1 | -0.261ns (n=21) |
| 3. Kin share no allele/ different diet vs non-kin share both alleles/ same diet | Diet 1 | -1.987* (n=17) |
| | Diet 2 | -2.086* (n=14) |

* denotes significant differences at $P < 0.05$, ns = $P > 0.05$ (not significant)

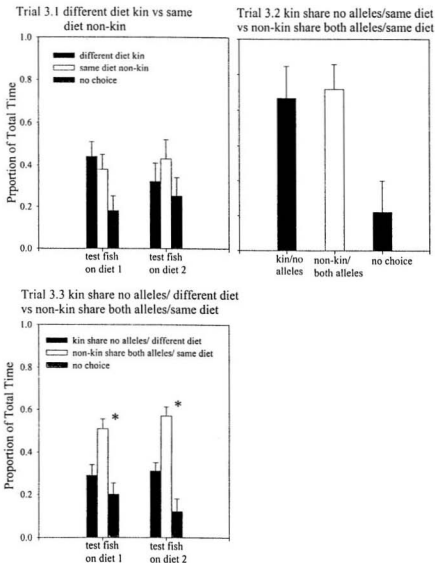


Figure 5.3 Proportion of total time spent in choice alleys and no choice area by the test fish in three trials in Experiment 3. Vertical bars = standard error, * denotes significant differences at $p < 0.05$.

5.4 Discussion

The results of this study suggest that the role of the MHC in influencing kin discrimination in juvenile Atlantic salmon is affected by a change in environmental (diet) cues. Dietary cues either supplant, rival or supplement genetic cues under different situations. When the test fish were given a choice between two kin groups, diet cues appear to supplant genetic cues. Juveniles preferred siblings with a similar diet regardless of whether they shared any alleles or not. When the juveniles had a choice between two non-kin groups with different MHC alleles, diet cues rival genetic cues. Juveniles did not have a preference for either non-kin sharing both alleles fed a different diet or non-kin sharing no alleles fed a common diet. However, when both dietary and genetic cues were opposite to kinship, juveniles preferred to associate with non-kin sharing both alleles and a common diet over kin sharing no alleles fed a different diet. In such situations diet cues supplement genetic cues in the recognition process.

Phenotypic variance of an individual is the combined effect of both genetic and environmental components (Falconer 1989). Grafen (1990) considered that variance in matching phenotypes is entirely attributable to variance in genotype, while Byers and Beckoff (1991) argue that differences in phenotype can be produced among genetically identical individuals by differences in developmental environment. The rearing environment does influence kin discrimination in some vertebrates including salmonids (Blaustein *et al.* 1987; Hiscock & Brown 2000; also see Chapter 2). At the chemical level of analysis in insect species, the ratio of different cuticular hydrocarbons vary as a function of genetic and environmental factors and can provide information for

recognition of colony, caste, age, class and other characteristics (reviewed in Todrank *et al.* 1998).

It is evident from kin selection theory that altruism should evolve more readily in those animals that can accurately identify kin and direct their altruistic acts exclusively towards them. However, most recognition systems have some degree of errors (Sherman *et al.* 1997). The two types of errors, recognition and rejection errors are analogous to type I and type II errors in statistics (Reeve 1989). In statistics, a type I error occurs when the null hypothesis is wrongly rejected, where as type II error occurs when the null hypothesis is wrongly accepted. In kin recognition, an acceptance error (type I error) occurs when an individual identifies a social partner as kin when it is non-kin, where as rejection error (type II error) occurs when an individual identifies a social partner as non-kin when it is kin.

Theoretically, the relative importance of genetic versus environmental cues in kin discrimination should optimize the balance between acceptance errors and rejection errors (Sherman *et al.* 1997). It is difficult to assess the relative importance of dietary and genetic cues because juveniles may be using different type of cues as different functions and act accordingly. They may interpret the information in the odour cues differently depending upon their situation or requirements. They probably use the MHC based genetic cues for kin recognition and the odours associated with diets for food selection (Hepper 1991). If an individual totally depends on environmental cues it might mistakenly favour non-relatives that share the same environment which increase acceptance errors. If an individual totally relies on genetic cues it might increase rejection

errors as all siblings may not share alleles. The optimal balance of these errors is predictable from the knowledge of the organism's environment, life history and its genetic system. Genetic cues are more useful for organisms that live in more homogenous environments such as sessile tunicates. Larval tunicates settle near and fuse with individuals that carry the same allele at a histocompatibility locus (Grosberg & Quinn 1986). Interactions with non-relatives are rare since the matched locus is highly polymorphic (Rinkevich *et al.* 1995) and hence the cost of acceptance errors is low (Sherman *et al.* 1997).

Salmonids occur in diverse environments. As the fry emerge from the redd, they can be swept downstream or to the periphery of the stream (Hutchings 1993). They would be likely to be mixed with differently related individuals (Brown & Brown 1993). Considering their life history, kin and non-kin individuals can occur in the same environment sharing similar diet, etc., and the cues they learn from the environment would not reliably correlate with kinship. Juveniles of Atlantic salmon and brook trout showed a bias behaviour towards non-kin sharing the alleles at the MHC and a similar diet. Considering the high polymorphism at the matched locus among salmonids, such discriminations are extremely rare in nature but such discriminations could outweigh any costs associated with the behaviour. The benefits to the individual involved in such discrimination could be sufficiently higher than accepting kin that did not share any alleles or a similar diet. Inclusive fitness benefits may be gained through kin association in shoals (Quinn & Busack 1985; Olsen 1989) or reduced aggression toward kin in the neighbouring territory (Brown & Brown 1992; 1993a; 1993b; 1996). Bias behaviour

towards non-kin would be likely to increase the individual's direct fitness (e.g survival of the individual) without increasing any indirect benefits (e.g., survival of kin) and hence, may represent a trade-off between an individual's direct fitness and indirect fitness.

The results also show that overlapping cues cause recognition errors. When one type of cue, either genetic or dietary, overlaps with unrelated individuals neither kin or non-kin was favoured, but when both cues overlap recognition errors occur by accepting non-relatives sharing dietary and genetic cues and rejecting relatives that did not share alleles or a similar diet. Sherman *et al.* (1997) suggest that recognition errors may persist because error-related costs of kin discrimination outweigh the benefits. If either rejection or acceptance errors become costly selection may favour universal acceptance or universal rejection of kin and non-kin (Reeve 1989). However, recognition errors due to discrimination of non-relatives are infrequent and the cost of acceptance errors can be low in nature. This is because these MHC loci are highly polymorphic and individuals that share similar alleles usually are close kin. At the same time rejection errors can be low because siblings are more likely to share alleles and a common diet than non-siblings and hence overlapping cues are rare in a natural context.

CHAPTER 6
EFFECT OF KINSHIP
ON GROWTH IN JUVENILE ATLANTIC SALMON
AND BROOK TROUT

6.1 Introduction

Some salmonids are territorial as juveniles and begin to defend foraging territories soon after emergence from the redd (Scott & Crossman 1973; Dill 1977; Gibson 1981; Scott & Scott 1988). Reduced frequency of territorial defense behaviours reduces the energy expenditure (Puckett & Dill 1985) and risk of physical injuries associated with such behaviours (Abbott & Dill 1985). As the fry emerge, they may be carried downstream or to the periphery of the stream by the currents (Hutchings 1993). As a result, Brown and Brown (1993a) suggest that there is a possibility of having either kin or non-kin as territorial neighbours. Based on Hamilton's (1964) theory on the evolution of social behaviour, individuals can increase their inclusive fitness by biasing their behaviour towards related versus unrelated conspecifics. This theory argues that by either cooperating with or not antagonizing kin, an individual can increase its own genetic fitness (Wilson 1987). Based upon this, Waldman (1988) predicted that individuals are expected to compete more intensely with unrelated individuals rather than siblings. However, this prediction is contrary to Walls and Blaustein's (1994) suggestion that patterns of resource utilization would be similar among related individuals or siblings because they are phenotypically similar, leading to intensified competition among close kin.

In salmonid juveniles the effects of kinship on growth remain controversial and the available data support both of the above predictions (Beacham 1989; Quinn *et al.* 1994; Brown *et al.* 1996). In Atlantic salmon and rainbow trout a significant reduction in aggressive behaviour was observed when their neighbours were kin compared with when the neighbours were non-kin (Brown & Brown 1993a). Arctic char being reared with kin has been shown to result in increased growth and reduced size variation (Brown *et al.* 1996). However, Beacham (1989) found the opposite results. He compared the mean and variance of growth rates in juvenile coho salmon reared as full sibling and mixed sibling groups and observed a higher variation in growth rates in full sibling groups with no overall difference in growth rate. Quinn *et al.* (1994) also reported similar results from a study conducted in an experimental stream channel using coho salmon. These results (Beacham 1989 and Quinn *et al.* 1994) suggest that fish from a fast growing and competitive family may grow faster or show less variation in growth when reared with members of other, comparatively less competitive families than when reared with highly competitive siblings.

This study was designed to examine the effect of social environment or kinship on growth in juvenile Atlantic salmon and brook trout. Based upon previous studies of salmonids I tested the null hypothesis that 'kinship has no effect on the growth in the two species'.

6.2 Methods

6.2.1 Test fish

Kin and non-kin groups of Atlantic salmon and brook trout were created as explained in Chapter 2. After hatching, fry were transferred to cylindrical 1 m³ rearing tanks. Kin and non-kin groups were reared separately. The fish were fed with salmon/trout starter feed (Vextra) and continued with the same feed until the start of the experiments and during the experiments. Two separate kin groups (two families) and one non-kin group of Atlantic salmon and one kin group (one family) and one non-kin group of brook trout were maintained. Each Atlantic salmon tank contained 120 fry while the brook trout tanks contained 90 fry (initial stocking density of 0.75 kgm⁻³). Juveniles were fed 1% mean body weight once per day. A sub-sample of 20 fish from each tank was selected arbitrarily. Fish were anaesthetized lightly using MS222 and were weighed (to the nearest 0.05 g) and measured the length (to the nearest 1.0 mm). The measurements were taken at 4, 8, 11 and 15 months post hatch. The water temperature ranged from 2-18°C during the study period. Mortality was recorded daily. Ambient fresh water was provided with a flow rate of 3 l/min. Fish were raised under a natural photoperiod through out the year.

Means of both weight and length data were analysed using one-way ANOVA (Sokal & Rohlf 1995). Individual comparisons between groups were done using Welch's approximate *t*-test for unequal variances (Zar 1984). Variance data were compared using a Bartlett's test of homogeneity of variance (Snedecor & Cochran 1989).

6.3 Results

Both mean weight (Figure 6.1 & Figure 6.2) and mean length (Figure 6.3 & Figure 6.4) were greater in the kin groups than in non-kin group both species. Significant differences were observed in mean weight and length data starting from the first eight months in both species. In Atlantic salmon between kin group 1 and non-kin group significant differences were observed in mean weight ($F_{1,38}=15.244$, $p<0.001$; $F_{1,38}=10.413$, $p<0.005$) and length ($F_{1,38}=9.535$, $p<0.005$; $F_{1,38}=6.241$, $p<0.05$) at 11 and 15 months respectively. Similarly significant differences were observed between the kin group 2 and the non-kin group (weight $F_{1,38}=35.381$, $p<0.001$; $F_{1,38}=22.007$, $p<0.001$; and length $F_{1,38}=30.031$, $p<0.001$; $F_{1,38}=16.258$, $p<0.001$) at 11 and 15 months respectively. A significant difference in mean weight was observed between the two kin groups at 11 months ($F_{1,38}=5.804$, $p<0.05$) and at 15 months ($F_{1,38}=7.831$, $p<0.05$) and in mean length at 11 months ($F_{1,38}=4.762$, $p<0.05$) and 15 months ($F_{1,38}=4.341$, $p<0.05$).

In brook trout a significant difference in mean weight was observed only at 15 months ($F_{1,38}=4.855$, $p<0.05$). Mean length differed significantly at 11 months ($F_{1,38}=4.267$, $p<0.05$) and 15 months ($F_{1,38}=8.668$, $p<0.05$).

A higher variance in both weight (Figure 6.5 & Figure 6.6) and length (Figure 6.7 & Figure 6.8) was observed in the non-kin groups in Atlantic salmon and brook trout throughout the experiment. Variance in weight and length between kin and non-kin groups were significantly higher in the non-kin group from 4 months in brook trout (Figure 6.6 & Figure 6.8). Similar differences were observed in the variance of Atlantic salmon length and weight starting from 8 months (Figure 6.5 & Figure 6.7). However, no

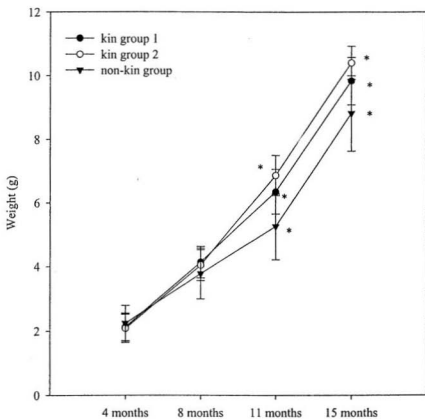


Figure 6.1 Mean weight (g) of juvenile Atlantic salmon in two kin groups and a non-kin group. Vertical bars = standard deviation, $n = 20$, * denotes significant differences at $p < 0.05$.

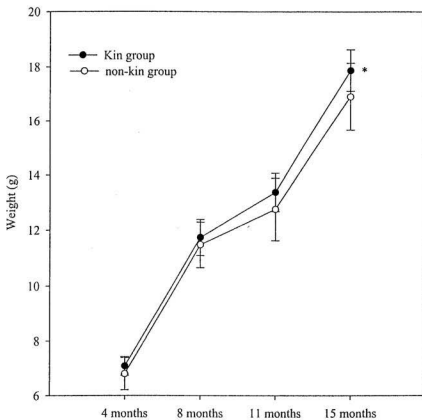


Figure 6.2 Mean weight (g) of juvenile brook trout in kin and non-kin groups. Vertical bars = standard deviation, $n = 20$, * denotes significant differences at $p < 0.05$.

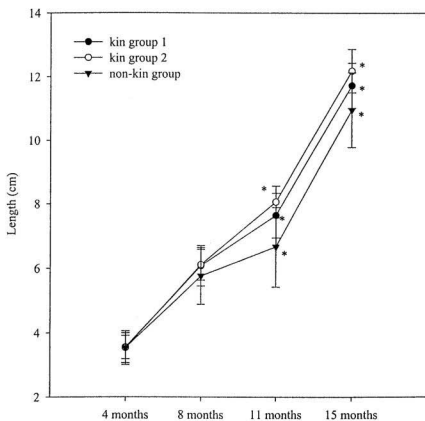


Figure 6.3 Mean standard length (cm) of juvenile Atlantic salmon in two kin groups and a non-kin group. Vertical bars = standard deviation, $n = 20$, * denotes significant differences at $p < 0.05$.

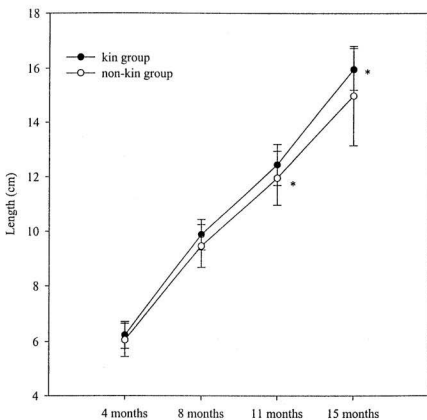


Figure 6.4 Mean standard length (cm) of juvenile brook trout in kin and non-kin groups. Vertical bars = standard deviation, $n = 20$, * denotes significant differences at $p < 0.05$.

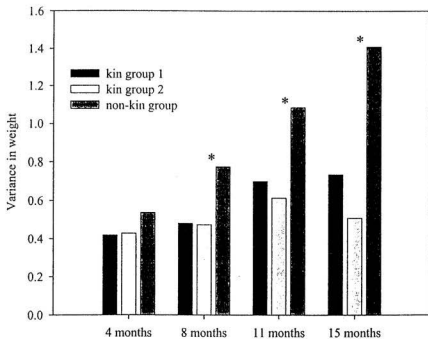


Figure 6.5 Variance in weight for Atlantic salmon in two kin groups and a non-kin group. * denotes significant differences at $p < 0.05$

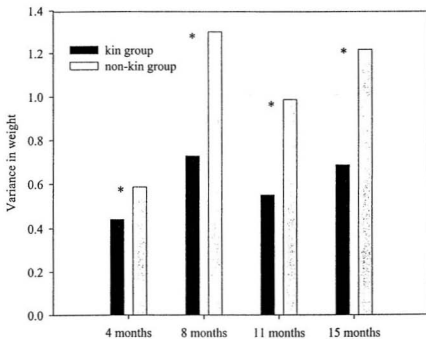


Figure 6.6 Variance in weight for brook trout in kin and non-kin groups.
* denotes significant differences at $p < 0.05$

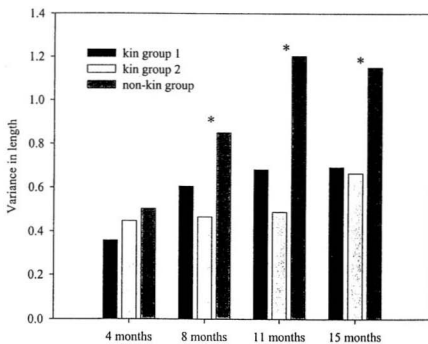


Figure 6.7 Variance in length for Atlantic salmon in two kin groups and a non-kin group. * denotes significant differences at $p < 0.05$

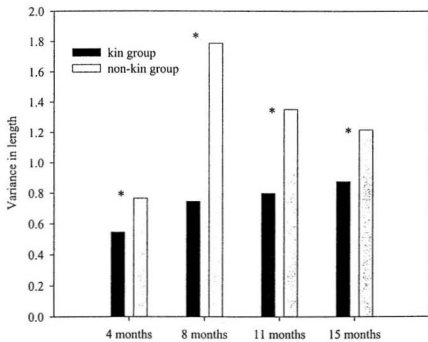


Figure 6.8 Variance in length for brook trout in kin and non-kin groups.
* denotes significant differences at $p < 0.05$

significant differences in length and weight variances were observed between kin group 1 and kin group 2.

6.4 Discussion

Due to lack of tank space I was unable to run adequate replicates for treatments in this study. The lack of replication compromises the generality of the results. However, as will be discussed, the results are in general agreement with previous studies.

A higher and a less variable growth was observed in the kin tanks compared to those of non-kin group in both Atlantic salmon and brook trout. Agonistic interactions are known to decrease in the presence of kin or familiar individuals over a broad range of taxa from mammals (e.g. Fuller & Blaustein 1990; Ylonene and Viitala 1990) to sea anemones (Francis 1973; 1988). If individuals in a kin group cooperate among themselves, sharing resources and displaying less agonistic interaction, it may lower mortality and result in higher and less variable growth. The results of this study and those of Brown *et al.* (1996) are consistent with this prediction where in juveniles higher mean and lower variance in growth was observed in kin tanks. In my study I used two kin groups of Atlantic salmon and a significant difference in the mean weight and length data was also observed between these kin groups (kin group 1 and kin group 2; Figure 6.1 & Figure 6.3). This difference in the growth between kin groups could be due to genotypic differences.

In an evaluation of the effect of kinship on growth performance, the best method to follow is to have the same kin group reared as one family and individuals from the

same family reared communally with unrelated individuals (e.g. Beacham 1989; Quinn *et al.* 1994). In this method, the expression of the genetic variation could be eliminated. In my study and in the study of Brown *et al.* (1996) separate males and females were used to create kin and non-kin groups. This rearing method did not allow the analysis of the performance of individuals from one family under the two different social environments (i.e. with kin as neighbours and non-kin as neighbours). I cannot make a generalized conclusion that observed differences in my study are entirely due to the effect of social environment (i.e. kin being more cooperative and less aggressive in a kin group) because I did not have the data to compare the individuals from the same family reared under a non-kin environment. The observed differences in growth between kin and non-kin groups could also be attributed to the genetic differences of the different families. Moreover, aggressive defense of feeding territories is characteristic of stream dwelling salmonids including Atlantic salmon (Stradmeger & Thorpe 1987) and brook trout, but not in standing habitats (Grant & Noakes 1988; Bachman 1984). The constraints on movement imposed by the size and the shape of the tank may have led to less aggressive or less cooperative behaviour than might occur in a natural stream condition.

The less variable growth of the kin groups compared to the non-kin group do not support the Beacham's (1989) and Quinn *et al.*'s (1994) finding (i.e. the growth of coho salmon reared in single families was more variable than the growth of the same families in tanks containing mixed families). Their findings support the prediction that more genetically similar individuals experience higher competition and are similarly efficient at obtaining resources, resulting in lower and more variable growth.

In studies on anurans carried out to determine how relatedness influenced individual performance in growth and development produced conflicting results. In some species growth is reduced in kin groups compared to that of non-kin group. Conversely, growth is enhanced in some species while in other it is unaffected when individuals are reared with kin. In two species (*Rana arvalis* & *R. cascadae*) growth is reduced in kin groups, compared to that of in non-kin group (Shvarts & Pyastolova 1970; Hokit & Blaustein 1997). However, growth is enhanced in kin groups of *Pseudacris triseriata* (Smith 1990) and in *Rana sylvatica* (see Walls & Blaustein 1994). Results with *Bombina variegata* and *Bufo americanus* are variable with growth either enhanced, inhibited or unaffected when individuals are reared with kin. Tadpoles of *Bombina variegata* grew more in kin groups than in groups of non-kin (Jasienski 1988). However, later studies on the same species showed opposite results (Hokit & Blaustein 1994; 1997; Walls & Blaustein 1994).

Anderson and Sabado (1999) investigated the effects of kinship on the growth of the kelp perch, *Brachyistius frenatus*, which do not exhibit overt aggressive or cooperative behavioural interactions. These authors revealed that average growth rates were similar between kin and non-kin treatments while the variation in growth increased initially in non-kin compared to kin. Based on their results they suggested a third alternative for the kinship effect on growth. These authors (1999) suggest that the equivalent rates of growth between groups of kin and non-kin and lower variation in growth among kin could simply reflect inherent genetic similarities in the absence of aggressive or cooperative behaviours. Absence of cooperative or agonistic behaviours in

this species has given these authors an opportunity to explore the effect of genetic relatedness independent from effects due to behavioural interactions.

CHAPTER 7

SUMMARY & CONCLUSIONS

The experimental work described in this thesis was based on Hamilton's kinship theory proposed in 1964. The first four chapters present imperial work regarding recognition cues of genetic and environmental origin in juveniles of Atlantic salmon and brook trout. In the second chapter I investigated the effect of diet as an environmental cue on the kin discrimination ability of both species. The third chapter dealt with techniques of isolation and characterization of MHC class II B1 locus and a brief survey of polymorphism of this locus within selected samples of Atlantic salmon and brook trout collected from four different areas in Newfoundland. Using this technique I analyzed the genotype of the kin and non-kin groups and studied the influence of MHC class II B1 locus on kin discrimination in both species. The results from experiment reported in Chapter 2 demonstrated that kin discrimination in Atlantic salmon and brook trout is influenced by dietary cues. Results from Chapter 4 provides evidence for the influence of MHC-based genetic cues in kin discrimination. In the Chapter 5 I examined the interaction of the genetic and environmental cues on producing discriminable odours used in kin discrimination in juvenile Atlantic salmon. Due to the lack of tank space I was unable to conduct the same experiments with brook trout. The data from Chapter 5 provided evidence that both environmental and genetic cues are equally important and the relevance of each cue is context dependent. The last experimental chapter (Chapter 6) examined the effect of kinship on growth and demonstrated higher and less variable growth in individuals when reared with kin compared to individuals reared with non-kin.

The data presented in this thesis provide answers to some of the questions addressed by Barnard (1990)- questions that are challenging in terms of both methods of investigation and interpretation and theoretical approaches:

If discrimination is taking place, how is it achieved? On what basis is discrimination taking place-matching at single loci, matching for overall phenotypic similarity? (Barnard 1990)

Earlier studies have shown that both Atlantic salmon and brook trout discriminate kin (Brown & Brown 1992; Hiscock & Brown 2000). The results of Chapter 4 demonstrate that the importance of the genetic similarity at the MHC class II B1 locus as the basis for kin recognition among juveniles of both Atlantic salmon and brook trout. Juveniles of both Atlantic salmon and brook trout showed a preference for kin sharing both alleles to kin sharing no alleles, similarly, test fish chose non-kin sharing both alleles over non-kin sharing no alleles. The preference for individuals sharing alleles demonstrates that discrimination is enhanced when matched at single locus. Data from the same study provide evidence for matching of the overall phenotypic similarity during discrimination. Juveniles preferred kin over non-kin, when both groups did not share any alleles at the MHC, which suggests that kin discrimination is not simply taking place by matching genes at a single locus, but by matching phenotypic correlates of a combination of genes in the entire genome including the MHC. Juveniles showed no preference for kin sharing no alleles over non-kin sharing one or both alleles which provides evidence for the importance of matching of both the MHC locus and the overall phenotypic similarity for discrimination. If discrimination is based on one particular locus alone, the

similarity at other loci is irrelevant and hence unrelated individuals bearing the same alleles should be treated in the same way as those sharing them by common descent.

What roles does kinship per se play in discrimination? Is kinship, for example, a rule of thumb for distinguishing allele cobearers or a rule for estimating genetic similarity? (Barnard 1990)

The results of Chapter 4 further indicate that MHC based kin discrimination in the two salmonid species does not distinguish allele cobearers. Juveniles of both Atlantic salmon and brook trout did not choose non-kin sharing the MHC allele over kin sharing no alleles. Data presented in Chapter 3 indicated that a high level of polymorphism occurs at the allelic level and that the amino acid difference is maintained at the MHC class II B1 locus in the two species. In nature, non-kin sharing both alleles could be very rare. However, individuals can share alleles without being a close relative. The hypothetical 'green beard' -type recognition (Dawkins 1976) is a mean of recognising allele cobearers (Barnard 1990). It is a recognition system that is independent of kinship by common descent (Dawkins 1976; Rushton *et al.* 1984; Waldman 1987), though some authors (Holmes & Sherman 1983; Hepper 1987; Fletcher 1987) discuss it in the context of kin discrimination. Hamilton (1987) suggests that the use of single loci in historecognition appears to bear some resemblance to green beard discrimination. A tunicate study by Grosberg and Quinn (1986) showed that larvae settle with an unrelated colony that carried the similar histocompatibility allele is an example of discriminating allele cobearers.

As a result of independent assortment at meiosis and random association of gametes at fertilization, some members of a kin group may actually have a large proportion of their alleles than do others. In a diploid organism, $\frac{1}{4}$ of members of a sibling cohort will share the same alleles, $\frac{1}{2}$ will share only one allele and $\frac{1}{4}$ will have completely dissimilar alleles. If kin groups are genetically highly variable, recognition of genetically more similar and less similar individuals might be possible. I examined whether individuals can estimate the genetic similarity at the MHC locus during discrimination and found that kin sharing both alleles were preferred over kin sharing no alleles and similarly non-kin sharing both alleles were chosen over non-kin sharing no alleles. However, these observations cannot be generalized to answer the second part of the question as to whether kin discrimination is estimating genetic similarity. The ability to discriminate between kin sharing no alleles vs kin sharing one allele and kin sharing both alleles vs kin sharing one allele were not studied.

What are the decision rules for expressing discrimination? How does the expression of discrimination vary with individual phenotype? (Barnard 1990)

Data presented in Chapters 2, 4 and 5 together show that the combined effect of both genetic and environmental components is important for expressing discrimination. The mechanism for kin recognition in salmonids is phenotype matching (Winberg & Olsen 1992; Brown *et al* 1993). The phenotype of an individual combines the genetic relatedness and/or the other gene(s) with environment effects (Falconer 1989). Data presented in Chapter 2 showed that the rearing environment influence discrimination. Juveniles could not discriminate between kin and non-kin when kin were fed with a

different diet and non-kin shared a common diet with test fish. Diet cues appear to mask the perception of kin related genetic cues or provide an alternative attractive stimulus of the location of a preferred food source. Chapter 4 showed that expression of discrimination also varied with the sharing of alleles at the MHC. When non-kin shared alleles and kin did not juveniles could not discriminate kin. The results of Chapter 5 suggest that the effect of the MHC in kin discrimination in juvenile Atlantic salmon is also affected by the change in environmental (diet) cues. When unrelated conspecifics shared a common diet and shared alleles, juveniles preferentially associated with non-kin. In nature salmonids occur in diverse environments. When unrelated individuals occurred in the same environment (share same diet) and share alleles at a matching locus, they are preferred over kin that did not share any alleles or a similar environment.

What are the fitness consequences of discrimination? (Barnard 1990)

Results in the Chapter 6 showed that a higher mean growth and a less variable growth was observed in the individuals reared in kin groups compared to those of non-kin groups in both Atlantic salmon and brook trout. Agonistic interactions are known to decrease in the presence of kin in some salmonids (Brown & Brown 1993a). Increased growth is a benefit of kin discrimination as it increases the potential for overwintering survival has been shown in previous studies (see Brown & Brown 1996b).

Kin-biased behaviour represents a trade-off between direct and indirect fitness benefits to an individual (Brown & Brown 1996a). Direct fitness is the individual's own reproductive success while indirect fitness is the reproductive success of kin. Results in Chapter 5 shows that individuals may select non-kin if they occur in the same

environment sharing similar resources and sharing alleles at the MHC. If it is beneficial to be with non-kin (i.e share common environment) which increase survival then this would increase the direct fitness of the individual. However, no indirect benefits occur from these associations. Such discriminations represent a trade-off between an individual's direct fitness and indirect fitness.

How does discrimination relate to ecology and life history? (Barnard 1990)

It is difficult to draw a general conclusion regarding how these results relate to the ecology and life history of the two salmonid species studied. However, the observed results are consistent with the life history of both Atlantic salmon and brook trout. Majority of salmonid fishes return to their natal site to spawn. Therefore the individuals that occur in a stream could have varying degree of relatedness. As the fry emerge from the redd, they can be swept downstream or to the periphery of the stream and there they adopt feeding stations and establish and defend territories (Hutchings 1993). They would likely be mixed with differently related individuals (Brown & Brown 1993a). Environmental cues alone would unlikely serve as reliable cues that correlate with kinship because juveniles of one family can occur in different parts of the stream feeding on distant diets. On the other hand genetic cues alone would be unlikely to provide reliable cues because unrelated individuals also can bear the alleles. Environmental cues and genetic cues together serve an accurate assessment of kinship.

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